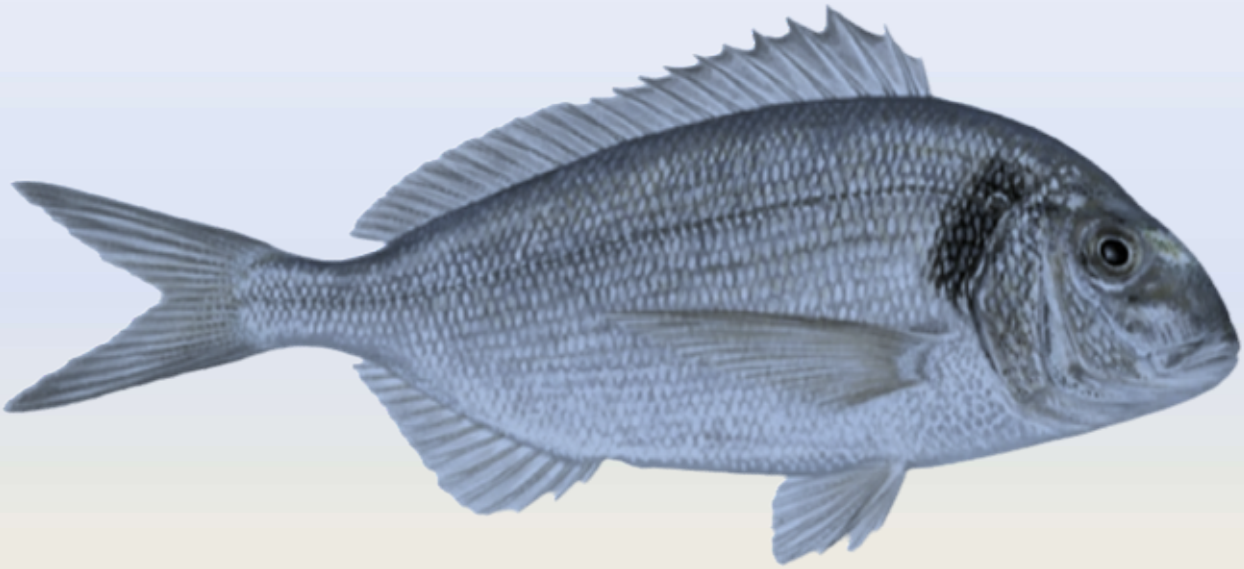
UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

Grupo de Investigación en Acuicultura

ECOAQUA

Effect of Nutritional Programming through Broodstock Diets and Selective Breeding on Reproductive Performance and Offspring Quality of Gilthead Seabream (*Sparus aurata*)



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Doctoral Thesis

University of Las Palmas de Gran Canaria
Spain

EFFECT OF NUTRITIONAL PROGRAMMING THROUGH BROODSTOCK DIETS AND SELECTIVE BREEDING ON REPRODUCTIVE PERFORMANCE AND OFFSPRING QUALITY OF GILTHEAD SEABREAM (*SPARUS AURATA*)

Ferosekhan Shajahan

PhD Thesis 2022

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

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LIST OF ABBREVIATIONS

AA	Amino acid
AChE	Acetylcholinesterase
ALA	Alpha linolenic acid
ARA	Arachidonic acid
BHT	Butylated hydroxytoluene
BPG	Brain-Pituitary-Gonad Axis
cDNA	Complementary DNA
cox2	Cyclooxygenase-2 gene
CVD	Cardiovascular diseases
cyp19a	Gonadal aromatase promoter
dah	Days after hatch
ddPCR	Digital droplet polymerase chain reaction
delta 5; FADS1	Delta-5-desaturase enzyme
delta 6; FADS2	Delta-6-desaturase enzyme
DHA	Docosahexaenoic acid
E_2	17β-estradiol
EFA	Essential fatty acids
elovl5	Elongation of very long chain fatty acids protein 5 gene
elovl6	Elongation of very long chain fatty acids protein 6 gene
EPA	Eicosapentaenoic acid
FA	Fatty acids
fads2	Fatty acid desaturase 2 gene
FAME	Fatty acid methyl esters
FID	Flame ionization detector
FM	Fish meal
FO	Fish oil
FSH	Follicle-stimulating hormone
GH	Growth hormone
GLC	Gas-liquid chromatography
GnRH	Gonadotropin releasing hormone
GSB	Gilthead seabream
GSI	Gonadosomatic index
HD	High *fads2* expression
HG	High growth
HSI	Hepatosomatic index
KCl	Potassium chloride
KT	Ketotestosterone
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acids
LD	Low *fads2* expression
LG	Low growth
LH	Luteinizing hormone
LO	Linseed oil
MUFA	Monounsaturated fatty acids
PA	Palmitic acid

PBCs	Peripheral blood cells
PCR	Polymerase chain reaction
PG	Prostaglandins
PL	Phospholipids
ppara	Peroxisome proliferator-activated receptor alpha gene
PPARs	Peroxisome proliferator activator receptors
RO	Rapeseed oil
RPM	Revolutions per minute
SD	Standard deviation
SFA	Saturated fatty acids
TAG	Triacylglycerols
TFA	Total fatty acid
TST	Testosterone
VO	Vegetable oil

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere thanks and gratitude to my supervisors Prof. Marisol Izquierdo and Prof. Sachi Kaushik for the invaluable help, guidance, time, patience, kind advices and suggestions not only for my research work but also for my well-being throughout my PhD programme. I always grateful to both of you for your whole-hearted support and constant encouragements for successful completion of my thesis. It would not have been possible to complete this research work without your continuous encouragement and moral support.

I would always remain indebted to Prof. Daniel Montero for being our PhD coordinator and guiding me for the experimental design, providing the experimental facilities and helping in university formalities and document preparation.

I wish to express my heartfelt gratefulness to Prof. Hipolito Fernandez-Palacios, Prof. Juan Manuel Afonso, Prof. María Jesús Zamorano, Prof. Carmen Maria Hernandez-Cruz, Prof. Anselmo Gracia, Dr. Javier Roo and all the faculties in the ECOAQUA.

I am extremely thankful to Prof. Ana Gomez for the guidance and also arranging my research stay at IATS, CSIS, Torre de la Sal, Spain. I thank Mrs. Sol, Technician for helping in the lab analysis at IATS. I also thank Dr. Alicia Felip and Prof. Jaume Pérez Sánchez at IATS for your encouragement and support provided during my stay at IATS.

I am also thankful to Dr. Grethe Rosenlund and Dr. Rámon Fontanillas from Skretting Aquaculture Research Centre, Stavanger, Norway for your help in feed formulation and preparation.

My sincere thanks to all the staffs of GIA. I thank Natalia, Carmen Quintana, Guacimara, Andrea, Marco, Juan, Nabil, Rayco, Ruben and a special thanks to Lorena for the kind support provided for the broodstock studies.

Thanks to my friends at ULPGC, Alvaro Lorenzo, Cathaysa, David, Samira, Marta, Raquel, Toni, Suk, Alvaro Montero, Adnane. I thank specially to Reda for your kind support and help in arranging my stay, travel, NIE etc.

I always grateful to my teammates and my special thanks to Serhat and Hanlin for your remarkable help and support throughout my studies. You guys helped me lot and without your support I would have not able to complete my research studies. My cute and beloved juniors Siva and Yien, thanks for all your support and help. Thank you Sivaguru for being supporting me till date.

I thank all my batchmates friends from India, Antony Jesu Prabhu, Ramalakshmi, Naveen, Saro, Ram, Manikandan, Dhamu, Sathish, Sankar, Thinish, Uday, Govind for extending their helping hands and encouraging words. Especially, no words to describe the support, appreciation and care by my friends.

My heartfelt thanks to my seniors and juniors, Sivaraman, Vicky, Sekar, Loveson, E. Suresh, Saravanan, Biju Sam Kamala, Ajith Stalin, Mohan Raj, Ajay Anand, Ranjith, Jana, Chandrasekar, Arun Sudhagar, Lings, Velus, Radhakrishnan, A. Karthik, Sivaramakrishnana, Sadru, Sri Hari, Shamna, Grace Angel, E. Prabhu, Yuvarajan, Gogul, Ajith.

I am very much thankful and express my sincere gratitude to my senior colleagues at ICAR, Dr. J.K. Jena sir, Dr. P. Jayasankar sir, Dr. A.K. Sahu sir, Dr. B.R. Pillai madam, Dr. S.K. Sahoo sir, Dr. S.K. Swain sir, Dr. S.S. Giri sir, Dr. B.K. Das sir, Dr. P.K Sahoo sir, Dr. S.C. Rath sir, Dr. J.K. Sundaray sir, Dr. P.C. Das sir, Dr. Barman sir, Dr. Subodh Gupta sir, Dr. N.P. Sahu sir, Dr. A.K. Pal sir, Dr. Das Gupta sir, Dr. P.N. Ananth sir and to all my senior colleagues.

My thanks to my friends and seniors at CIFA, Sivaraman, Avinas, Suhas, Mukesh, Uday, Nitish, Rakhi, Snata, Puspa, Murmu, L. Sahoo, Rajesh Kumar, Shailesh, Panda, Sarkar, Pankaj, Sidd, Sunil, Mohan, Priyanka, Sweta, S.N. Sahoo, Rakesh, Anirban, Kiran, Anantharaja.

My deepest love and regards to my wife Arshiya, my son Rayyan for your sacrifice and moral support and encouragements. My strength is my family. Their support is always special to me. Thanks to all of you, my father Shajahan, my sister Banu, Ammu, my brothers jaila, abbu, riaz, my chitti Namisa, my nanny, my uncle Shahul, Aejaz, Munnu, and my in-laws and I always miss my mother Begamjan and her blessings and I miss my nanna too.

I thank to ICAR, Govt of India for providing such a nice opportunity and PhD fellowship to travel to Spain and to pursue my PhD and learn new skills and knowledge to widen up my capabilities. Finally, I thank each one who supported me and encouraged me to reach my passion. I love my Spain. Miss a lot my Las Palmas. Muchas gracias!!

Funding

Indian Council of Agricultural Research (ICAR), New Delhi, India for providing Doctoral fellowship.

ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India for providing study leave and necessary permission.

University of Las Palmas de Gran Canaria, Spain - Doctoral study – Host University.

PerformFISH project - European Union's Horizon 2020 research, innovation program under grant agreement No 727610 for research programme funding.

AquaIMPACT project - European Union's Horizon 2020 (Blue Growth) H2020-BG-2018-818367 for research programme funding.

PROGENSA III project - (Mejora de la competitividad del sector de la dorada a través de la selección genética, JACUMAR program with support from the European Maritime and Fisheries) for research programme funding.

I dedicate to Prof. Marisol & Prof. Sachi Kaushik....

CHAPTER 1

GENERAL INTRODUCTION

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1.1 Gilthead seabream biology and aquaculture production potential

Aquaculture is the fastest growing animal producing sector in the world, it is expected to play an important role in global food supply and it is projected to reach US $ 377 billion by 2025 (FAO, 2020; AMR, 2021). Fish do not only represent an excellent source of protein, amino acids and minerals, but are also a unique and rich source of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) and both these fatty acids are key dietary components for human health. These n-3 fatty acids have beneficial effects on neural development in young children and in the prevention of cardiovascular and inflammatory diseases, as well as neurological disorders (Gogus and Smith, 2010). The ever-increasing demand for seafood has resulted in shifting from conventional farming to intensive fish production (FAO, 2020). Such intensification should be long term and have minimum negative consequences for the environmental and natural resources (Klinger and Naylor, 2012). Over the years, awareness of the importance to include sustainable raw feed ingredient resources in fish feeds has increased. Over the last decade, reducing the amount of fish meal (FM) and fish oil (FO) use in the fish feed has become standard practise in order to ensure that aquaculture is an environmentally friendly and sustainable food production sector capable of providing a nutrient-rich diet for human consumption. There is a perpetual pressure on aquaculture industry to reduce its dependence on marine-based ingredients such as FM and FO (Welch et al., 2010). Both ingredients have become scarce in recent years, and the prices, which have climbed steadily over the last three decades, are projected to rise even more as demand continues to rise. As a result, a reduction in the usage of FM and FO is very much needed for the sustainable development of aquaculture. The incorporation of plant products at high levels in fish diet is recognized to have some disadvantages, particularly related to the differences and deficiencies of few or selected amino acid (AA), fatty acid (FA) and minerals composition compared to marine ingredients, but also to the presence of anti-nutritional factors in plant ingredients. Consequently, the replacement levels of marine ingredients must be adapted depending on the species and the developmental stages of fish. These differences in the composition of plant ingredients can have metabolic and physiological consequences that deserve further

investigation to provide adequate background for successful greater use of plant feedstuffs in aquafeeds (Gatlin et al., 2007).

Much progress has been made in the past many years for the partial replacement of marine FM and FO by plant ingredients in nutritionally well-balanced diets (Oliva-Teles et al., 2015; Tacon et al., 2015). Many studies have reported the complete replacement of FM by alternative plant protein sources in diets for both freshwater and marine fish (Tacon et al., 2015; Kaushik et al., 2004; Torrecillas et al., 2017) and FM replacement was somewhat achieved for fish. However, total replacement of FO in the diets of marine fish is difficult due to the non or lesser availability of n-3 LC-PUFA which is very much essential for marine teleost (Izquierdo, 1996; Tocher et al., 2010). Fish oil is a naturally rich source of n-3 LC-PUFA, despite some dissimilarities in the fatty acid profiles of different fish oils. Global availability of "fish oils" is very limited and therefore it must be utilised in a sustainable way. Vegetable oils (VO) have been used to partially replace FO either alone (Torstensen et al., 2008) or in combination with FM replacement (Liang et al., 2014; Simó-Mirabet et al., 2018). Marine fishes have a limited capacity for bioconversion of 18 carbon (18C) fatty acids to LC-PUFA, such as EPA and DHA, and the replacement of FO by VO is limited due to the paucity of n-3 LC-PUFA in VO, despite their high concentrations in 18 carbon fatty acid precursors. The initial step in LC-PUFA biosynthesis in fish is catalysed by delta 6 fatty acid desaturase (Δ6 Fads) enzyme, which inserts an extra double bond in the 18C precursors, linoleic acid (LA, 18:2n-6) or alpha-linolenic acid (ALA, 18:3n-3) to initiate the process. Therefore, this enzyme produces 18:3n-6 and 18:4n-3 and, subsequently, longer carbon chain fatty acids and essential fatty acids (EFA), such as the EPA (20:5n-3) and DHA (22:6n-3), are produced in fish after several elongation, desaturation, and β-oxidation steps (Monroig et al., 2011; Tocher et al., 2015). Marine teleosts have a low expression of fatty acyl desaturase gene (*fads2*), the gene that codes for Δ6 Fads enzyme (Tocher et al., 2015) and therefore, n-3 and n-6 LC-PUFA must be included in the diet.

Gilthead seabream (GSB) is a marine teleost which belongs to the Sparidae family that consists of large number of species with several genera. Sparids have wide geographical distribution and are found throughout the tropical and temperate waters and are also found in cold and brackishwaters. GSB (Figure 1-1) has an oval body shape, deep and compressed body, with a curved head and small eyes. It is a benthopelagic species and found generally in inshore waters and it is an euryhaline and shoal forming species (Sola et al., 2006). GSB is a carnivorous species, predominantly feeds on crustaceans, molluscs, insects, fishes and also its feeds on sea grasses and other aquatic plants. The reproductive biology of the GSB is reported

as a protandrous hermaphrodite, in the initial age period, being a functional male in the first two years and turning into females from the age of two years. Spawning occurs mostly during December to April month, when water temperatures reaches at 13-17 °C. GSB is largely farmed in cages with the production density of 15-25 kg m^3 and the culture period usually takes between 1.5 to 2.0 years to reach 400-600 g in commercial production condition. The nutritional requirement of major nutrients of GSB is only recently available and this information is vital for formulation and preparation of commercial diets for this species. In many studies, it was reported that GSB grow-out diets formulated and prepared to contain 45–50% crude protein and 18-22% crude lipid. As for most marine fish, FM and FO ingredients have been reduced in commercial diets being replaced by vegetable sources (Oliva-Teles, 2000; Gómez-Requeni et al., 2004).

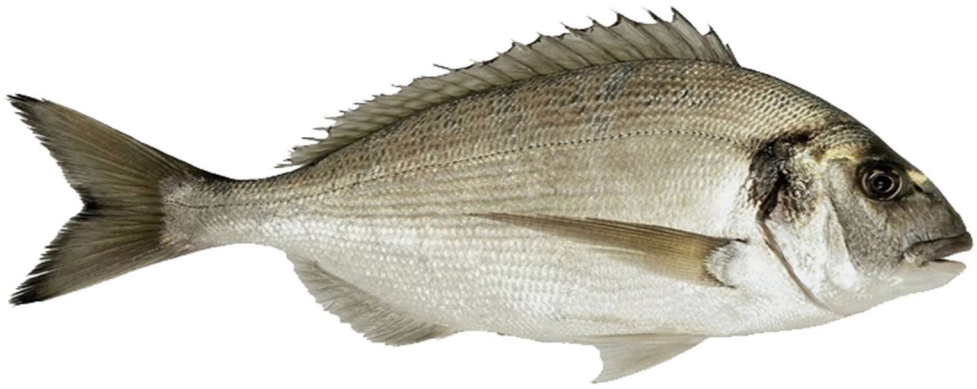

Figure 1-1. Adult specimen of gilthead seabream, *Sparus aurata.*

The GSB total production in 2018 was estimated around 200,000 tonnes (FAO, 2020) (Figure 1-2). In 2016, more than 90% of the GSB aquaculture took place in just 5 out of 20 countries, with Turkey being the leading producer (31%), followed by Greece (26%), Egypt (14%), Tunisia (7%) and Spain (5%). This species is also being cultured in many other Mediterranean countries, including Cyprus, France, Portugal, Croatia, Malta, Albania, Bosnia, Libya, Algeria, and Morocco (FAO, 2020).

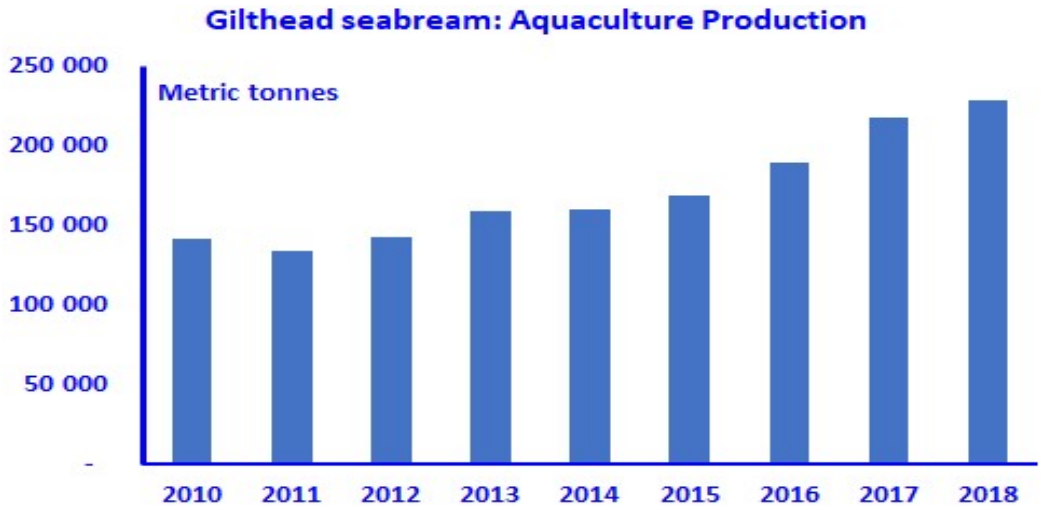

Figure 1-2. Gilthead seabream global aquaculture production data (Source: FAO, 2020).

1.2 Gilthead seabream reproduction

GSB is a batch and continuous spawner, and spawning takes place between November to June, corresponding to natural water temperatures of 13-19 °C. Females are daily spawners producing 20,000-40,000 eggs per kg body weight per day for a period of three months and the spawning is mostly dependent on the temperature and photoperiod. GSB spawn readily in captivity under optimal environmental and dietary conditions (Izquierdo et al., 2015; Xu et al., 2019; Ferosekhan et al., 2020a).

1.2.1 Endocrine control of reproduction

Fish reproduction is affected by various external or internal factors. The endocrine control of male and female fish reproduction is majorly influenced or controlled by the exogenous factors and endogenous physiological cycles, which regulates brain, pituitary, and gonadal functions (Zohar and Mylonas, 2001). The brain-pituitary-gonadal (BPG) axis and its mechanisms directly control the reproduction in higher vertebrates and chordates (Weltzien et al., 2004). In brief, gonadotropin-releasing hormones (GnRH) are produced by a specific group of GnRH neurons in the brain, which directly control or influence the pituitary through the production of two gonadotropin hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both hormones enter in the blood circulation and reach the steroidogenic cells in the ovary and testis (follicular cells in the oocytes and Sertoli cells in the testes). They in turn stimulate the gonadal development and maturation by stimulating the production of sex steroid hormones and several growth factors to support gamete production (Weltzien et al., 2004).

1.2.2 Sex steroids

In fish, spermatogenesis and oogenesis are primarily regulated by the several sex steroids and other factors (Fostier et al., 1983). The gonads are the final action point of the reproductive cycle, and it involves in two main functions, germ cell development (spermatogenesis and oogenesis) and steroidogenesis production in vertebrates. Spermatogenesis and oogenesis mark the beginning of puberty and subsequent reproductive cycles. Sex steroid hormones, such as testosterone and 11 keto-testosterone (11-KT) in males and 17β-estradiol (E2) in females, have crucial roles in the stimulatory and regulatory effects at the gonadal level for gamete production and also it acts as the feedback mechanisms in brain and pituitary to stimulate or inhibit the release of gonadotropins, LH and FSH in fish (Weltzien et al., 2004; Young et al., 2005).

1.2.3 Male reproduction

The endocrine regulation of spermatogenesis and spermiation in GSB is not well studied, but the information exists for the red seabream (RSB; *Pagrus major*) a close relative species to GSB (Teong, 1991). It was reported in RSB that during the month of June, spermatogonial germ cells present in the testis and in the month of November during early spawning season, spermatocytes and spermatids begin to appear, and during December (beginning of spawning season) spermatozoa appears in the testis. It was observed that testis development and sperm cell production was observed during January to April. The mRNA levels of LH β-subunit are recorded at high levels throughout the reproductive cycle, from the beginning of gametogenesis to the spawning season, and it tends to decrease rapidly to lower levels after the spawning season in RSB and same trend has been observed for the GSB (Teong, 1991).

1.2.4 Female reproduction

The GSB is a continuous batch spawner with an asynchronous ovary and its spawning season occurs between November to June, 4-6 months periods (Zohar & Gordin, 1979). The development of radioimmunoassay technique for GSB gonadotropin, LH and FSH and the molecular cloning of LH and FSH subunits of GSB has helped to study the kinetics and dynamics of the gonadotropins and other reproductive hormones during gametogenesis process (Zohar et al., 1990). The gonadal development, oocyte maturation, ovulation, and spawning are influenced by the LH, FSH and 17β-estradiol (E_2) sex hormones in GSB (Zohar et al., 1995). The oocyte maturation and ovulation are induced by the GnRH secretion that stimulate

the LH and FSH release (Gothilf et al., 1997). The continuous and sustained surge in E_2 levels is suggested to be responsible for stimulating vitellogenesis in earlier developing follicles for the production of vitellogenin by the liver for uptake by the growing oocytes (Zohar et al., 1995).

1.3 Genetic improvement in gilthead seabream

The application of genetic improvement research in animals and plants has made a substantial contribution to a sustain growth and improved productivity (Gjedrem et al., 2012). However, when compared to terrestrial farm animals, the application of the principles of quantitative genetics in fish has been limited and need more factors to be considered. Several breeding programs have been developed for Atlantic salmon (*Salmo salar*), trout (*Onchorhynchus mykiss*), tilapia (*Oreochromis niloticus*), catfish (*Ictalurus punctatus*), seabass (*Dicentrarchus labrax*), turbot (*Psetta maxima*) and gilthead seabream (*Sparus auratus*) (Gjedrem, 1997; Afonso et al., 2012). Selective breeding programme has various methods and approaches such as simplest mass selection to index selection methods and marker-assisted selection for production of offspring with desired traits. All the selection methods employed in fish breeding programme majorly use mass spawning in order to produce large quantities of the desired progeny (Chavanne et al., 2016; Jansen et al., 2017). The mass spawning programme has certain limitations in the selection process, since it lacks techniques to identify the individual offspring's parents to know the better performing parents. In order to overcome this issue in mass spawning, microsatellite markers are used as a powerful tool to identify and assign the parental contribution and performance through offspring performances (Navarro et al., 2009; Lee-Montero et al., 2013).

Broodstock selection in fish has been based on somatic growth (Navarro et al., 2009; Vu et al., 2019; Ye et al., 2017; Fernandes et al., 2017) , feed efficiency (DeVerdal et al., 2018; Besson et al., 2019), disease resistance (Wiens et al., 2018; Palaiokostas et al., 2016), deformities (Negrin-Baez et al., 2015; García-Celdrán et al., 2016), fillet yield (Vandeputte, et al., 2019; Schlicht, et al., 2019) or fillet fatty acid composition (Horn eta la., 2018; Prchal et al., 2018). Selective breeding programs in GSB have also addressed improved growth performance, disease resistance, skeletal deformities and carcass quality (Borrell et al., 2011; Piazzon et al., 2020; García-Celdrán et al., 2016; Lorenzo-Felipe et al., 2021). Assignment of parental contributions of better performing progenies of GSB has been assessed using a multiplex PCR technique (Borrel et al., 2011). In the past few years, the genetic improvement programs in GSB drawn major attention for the production of quality seeds and market size

fish in a sustained way. In Europe, it was reported that 30-45% of GSB seed comes from the genetic selection processes (Chavanne et al., 2016). It is majorly undertaken for growth performance and morphology as selected traits (Janssen et al., 2017), and genetic improvement per generation is in the range of 5-29% (Knibb et al., 1997; Brown et al., 2005). GSB broodstock selection was performed by the Best Linear Unbiased Prediction (BLUP) methodology for the somatic growth trait and has been widely used in commercial hatcheries (Fernandes et al., 2017). In addition to that, carcass, flesh and fillet quality, disease resistance and skeletal deformities are used as the major criteria in a series of joint projects among commercial and public research hatcheries in Spain through the PROGENSA project (Navarro et al., 2009; Lee-Montero et al., 2013; Negrín-Báez et al., 2015; García-Celdrán et al., 2016).

1.4 Broodstock nutrition

The nutritional composition and quality of a broodstock diet plays an important role in the egg and larval quality of fish, which supports the production of better performing offspring (Bell et al., 1997; Bruce et al., 1999; Izquierdo et al., 2001). The fatty acids (FA) compositions of broodstock diet have a significant role in ensuring successful reproduction and survival of the offspring (Izquierdo et al., 2001). The importance of broodstock nutrition in the development of the gonads, sperm, egg quality and larval performance is widely recognized, and several studies have focused on defining the roles of key nutrients in the reproduction of GSB (Fernandez-Palacios et al., 1995; Scabini et al., 2010). The total fatty acid composition of fish eggs reflects dietary composition of the parental diet and the dietary essential fatty acids (EFA) such as specifically EPA and DHA, and their role in egg and larval development was well documented (Watanabe et al., 1984, Fernandez-Palacios et al., 1995; Bell et al., 1997; Bruce et al., 1999; Izquierdo et al., 2001, 2015).

The importance of dietary n-3 long chain-poly unsaturated fatty acids (n-3 LC-PUFA), such as EPA and DHA, for marine fish and its necessity of supplying sufficient amounts of these fatty acids for successful production of eggs and larvae is recognized for many marine fish species (Izquierdo et al., 2001; Watanabe and Vassallo-Agius, 2003). Marine fish contain large amounts of EPA and DHA in the phospholipids of their cellular membranes, and low dietary levels of EPA and DHA negatively affect fish physiology and behaviour, especially in marine fish species (Izquierdo et al., 2001). Therefore, this reduced levels of EFAs can affect endocrine system of fish since their derived eicosanoids regulate the steroidogenesis pathway (Sargent et al., 2003). During gametogenesis process, n-3 LC-PUFAs, such as EPA and DHA, are preferentially incorporated into ova for the normal embryo development (Bromage, 1995).

These EFAs, as well as arachidonic acid (ARA), are recognized as major FAs improving egg quality and offspring development (Harel et al., 1994, Bruce et al., 1997). The dietary inclusion of different oil sources and their fatty acid profile affect the reproductive performance in GSB. The dietary level of EFAs for GSB broodstock is provided in the Table 1-1. Recent work by our research group with GSB broodstock fed specifically designed diets rich in EPA and DHA have shown improved reproductive performance and production of high-quality eggs and larvae (Ferosekhan et al., 2020a; Xu et al., 2019; Izquierdo et al., 2015). In these studies, we observed a strong correlation between dietary FAs and egg FA composition in GSB. This clearly indicates that the dietary FAs have a strong positive role in improving the egg FAs composition which ultimately improve the egg and larval quality in GSB (Table 1-1). It can be observed in this Table 1-1 that the study undertaken for nutritional programming approach has used increased inclusion level of LA and ALA, as FAs precursors for biosynthesis of EFAs (Ferosekhan et al., 2020a; 2021; Xu et al., 2019; Turkmen et al., 2020; Izquierdo et al., 2015) but in the old studies the importance was given to EPA and DHA for direct supplementation of EFAs to improve reproductive performance in GSB. The reproductive performance of GSB through nutritional programming approach has significantly improved the egg and larval quality even with reduced inclusion of FM and FO in the broodstock diets. It was observed in our recent studies that FO replacement by the use of a blend or single use of either rapeseed oil (RO) or linseed oil (LO) has not affected the reproductive performance and offspring quality of GSB through nutritional programming approach by the broodstock selected for either high *fads2* expression or high growth trait and fed with VO diets or partial supply of FO in the diets (Ferosekhan et al., 2020a; 2021; Xu et al., 2019; Turkmen et al., 2020; Izquierdo et al., 2015). The reported spawning quality parameters of all these studies were in the range of previously published information of GSB broodstock (Scabini et al., 2010; Fernandez-Palacios et al., 1995).

Table 1-1 EFA contents in diets and eggs, as well as reproductive performance of gilthead seabream broodstock from the dietary treatments leading to best fish performance in previous studies

Sl. No	Dietary treatment	Diet linoleic acid (%TFA)	Diet linolenic acid (%TFA)	Diet ARA (%TFA)	Diet EPA (%TFA)	Diet DHA (%TFA)	Egg linoleic acid (%TFA)	Egg linolenic acid (%TFA)	Egg ARA (%TFA)	Egg EPA (%TFA)	Egg DHA (%TFA)	Relative fecundity per Spawning (eggs/ kg female / Spawn)	Fertilization (%)	Egg viability (%)	Hatching %	Larval survival %	Reference
1.	20%FO/80%RO with High growth trait broodstock-mass spawning	12.32	4.85	0.35	5.26	7.46	11.02	3.15	0.65	5.65	20.99	43176	96.0	74.4	93.4	58.3	Ferosekhan et al., 2022 (submitted)
2.	100%VO-High growth broodstock-mass spawning	13.49	9.58	0.28	4.81	6.23	11.81	4.96	0.6	5.14	17.76	28451	98.7	54.3	87.2	64.4	Ferosekhan et al., 2021
3.	20%FO/80%RO with High *fads2* selection	11.1	4.9	0.4	6.6	8.4	10.6	2.7	0.8	5.8	21.8	48040	76.2	66.4	90.2	76.3	Ferosekhan et al., 2020a
4.	VMFO (100FO)	12.2	1.5	0.8	10.7	11.3	12.6	1.5	0.9	7.3	17.1	32889	57.9	53.41	50.21	38.91	Xu et al., 2019
5.	30%FO-70%LO/HΔ	9.9	16.3	0.3	4.8	6.0	11.4	5.5	0.6	4.0	15.1	36790	91.8	62.2	91.4	49.7	Turkmen et al., 2020
6.	40%FO/60%LO	11.2	21.4	0.4	5.6	6.6	10.5	13.1	0.7	5.4	15.1	38000	94.3	77.5	94.1	82.8	Izquierdo et al., 2015
7.	Carotenoid 0.006%/n-3 HUFA 2.5 %	3.9	0.6	0.7	7.7	9.6	3.5	0.6	0.9	8.3	20.5	36000	98.0	83.6	95.0	86.2	Scabini et al., 2010
8.	n-3 HUFA 1.60%	3.1	0.5	0.5	6.2	4.1	1.7	0.2	0.4	2.0	6.3	42000	94.0	74.8	96.4	46.9	Fernandez-Palacios et al., 1995

1.4.1 Lipids and essential fatty acid requirements in fish

Lipids are a varied group of organic compounds that are characterized by their insolubility in water due to their chemical structure. Lipids can be broadly classified into two groups: polar and neutral lipids. Phospholipids (PLs) make up the majority of polar lipids, whereas trylglycerols (TAGs) make up the majority of neutral lipids. Lipids, together with proteins and carbohydrates, are the primary macronutrients needed to provide both vital nutrients for energy generation as well as building blocks for cell and tissue growth. Essential fatty acids (EFA) must be supplied by lipids in order for fish to satisfy their FA requirements for the better growth. EFAs are necessary for optimal function of cellular metabolism, as well as the maintaining the membrane structural integrity, reproduction, gamete quality, and immunological function in fish, and it is not produced independently by the marine fish (Tocher, 2003; 2015).

EFAs are important components of phospholipids, main constituents of cell membranes and involved lipoproteins. Moreover, ARA and EPA are known to be precursors of an important class of signalling molecules named eicosanoids, and are known to possess both anti-inflammatory and protective properties in prostaglandin synthesis (Bell et al., 1986). A high variability exists in specific EFAs requirements among species, in particular between freshwater and marine fish (Tocher, 2003). This difference is essentially related to the capacity of fish to bio-convert C18 FA into LC-PUFAs, and therefore to the distinct activity of two classes of enzymes implicated in this conversion process: elongases and desaturases. Elongases are responsible for the condensation of activated FAs with malonylCoA in the FA elongation pathway, while desaturases introduce a double bond in the fatty-acyl chain at the C3, C6 or C9 position from the methylic terminal group (Tocher, 2003). This ability for elongation and desaturation is considered to be more effective in freshwater fish than in marine fish. The marine food webs are generally characterized by high levels of n-3 LC-PUFA (EPA and DHA), which chiefly came from the presence of microalgae, diatom and plankton whereas these FAs are not found in large amounts in the freshwater food webs (Parzanini et al., 2020). On the other hand, freshwater food webs are rich in ALA and LA and therefore, the widespread ability of freshwater fish to convert 18C PUFAs to the biologically active 20C and 22C PUFAs may be the result of an evolutionary adaptation and a high evolutionary pressure to maintain the capacity to endogenously produce LC-PUFA in freshwater species (Tocher, 2003).

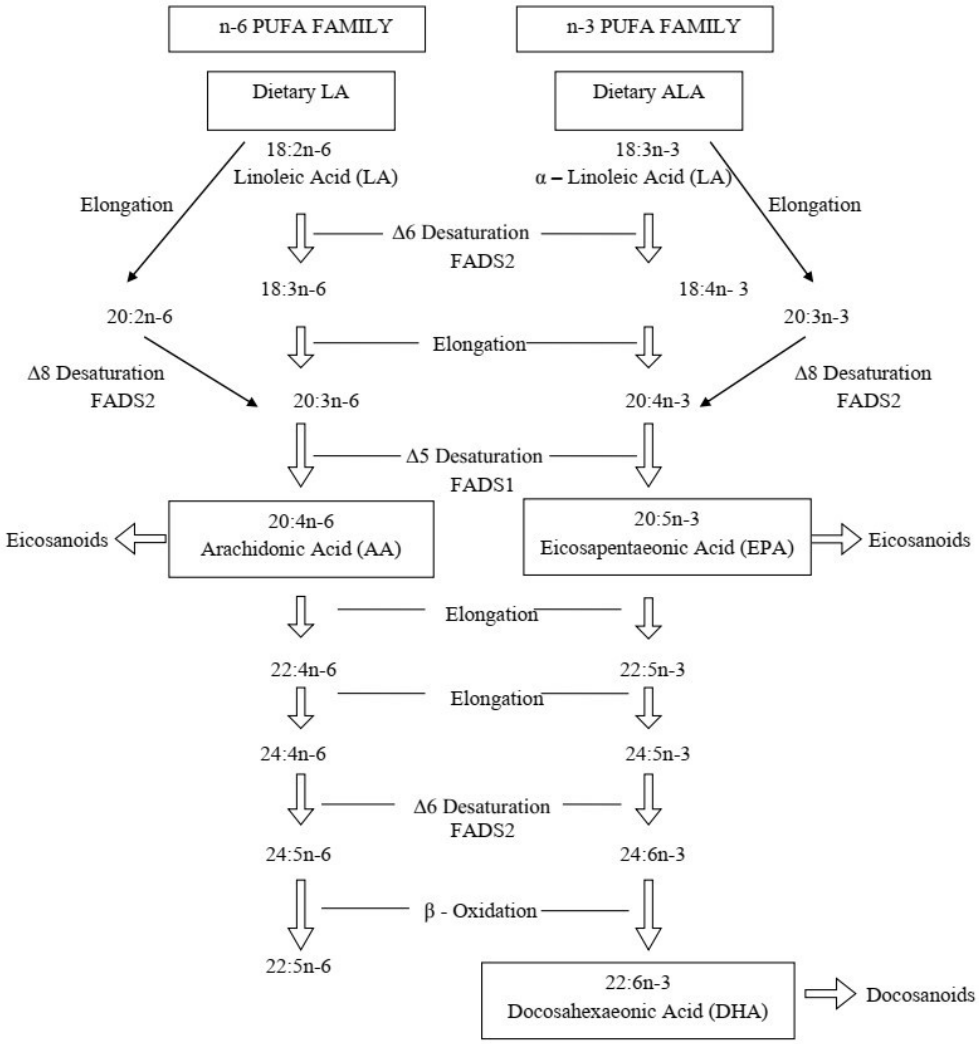

Figure 1-3. Endogenous biosynthesis pathway of n-6 and n-3 LC-PUFA from LA and ALA substrate through desaturation (Fads), elongation (Elvol) and β-oxidation in fish (Source: Ferosekhan et al., 2020b).

1.4.2 Endogenous biosynthesis of fatty acids and *fads* activity

The FAs supplied by the diet and those endogenously synthesized by the animal can, to a certain extent, be bio-converted to FA with longer or more unsaturated chains. In vertebrate, the liver represents the main site for FA synthesis through the FA synthetase complex through several steps of desaturation, elongation, and oxidation. Fish, as it is the case for all organisms, can *de novo* synthesize the saturated fatty acids 16:0 (palmitic acid) and 18:0 (myristic acid) in different proportions, depending on the species (Sargent et al., 2002). All fish are then capable to convert these two *de novo* synthesized FA into monounsaturated FAs (MUFAs), such as 18:1n-9, through the action of Δ9-desaturase. However, fish, as well as mammals and other

vertebrates, lack both the Δ12-desaturase and Δ15-desaturase, which are necessary to convert 18:1n-9 to 18:2n-6 and 18:2n-6 to 18:3n-3, respectively (Tocher, 2003). Thus, both 18:2n-6 and 18:3n-3 are EFAs and must consequently be supplied by the diet (Sargent et al., 2002, Tocher, 2003). These two FAs must be further converted into their LC-PUFA bioactive products: ARA of the n-6 series, and EPA and DHA of the n-3 series. As mentioned previously, the capacity to convert C18 PUFA to LC-PUFA varies within fish species, being high in freshwater and low in marine fish species (Sargent et al., 2002, Tocher, 2003). Synthesis of ARA from its C18 precursor requires a first step of Δ6-desaturation followed by an elongation to produce 20:3n-6, which is then desaturated to 20:4n-6 by the action of Δ5-desaturase. The synthesis of ARA then requires additional elongation and Δ6-desaturation steps, followed by a chain-shortening step in the peroxisome (Sargent et al., 2002).

The EPA fatty acid synthesis is attained by the action of Δ6-desaturation enzyme on 18:3n-3 to produce 18:4n-3, which in turn elongated to 20:4n-3, Eicosatetraenoic acid and followed by Δ5-desaturation process. The synthesis of DHA from EPA requires two further elongation steps, a second Δ6-desaturation and a peroxisomal chain-shortening step (Sprecher, 2000). The biochemical pathway of n-3 and n-6 LC-PUFA synthesis is depicted in Figure 1-3. Freshwater fish, including rainbow trout, carps and catfish are able to convert 18C (linoleic acid, 18:2n-6 and linolenic acid, 18:3n-3), into PUFAs. In marine fish, the biosynthetic activity of the LC-PUFA synthesis pathway seems to occur at a much lower level, or may even be entirely absent, compared to freshwater fish. At the molecular level, previous studies have shown that Atlantic salmon possess separate genes for Δ5 and Δ6 fatty acyl desaturases (Monroig et al., 2010). Distinct Δ6-desturase cDNA have been isolated from different fish species including freshwater and marine species (Seiliez et al., 2003, Zheng et al., 2004, Tocher et al., 2006). A bifunctional Δ5/Δ6-desaturases have been isolated from zebrafish, *Danio rerio* (Hastings et al., 2001) to identify their potential role in FAs biosynthesis. The fatty acid biosynthetic pathway comprises several steps catalysed by fatty acyl desaturases (Fads1-Δ5 and Fasd2-Δ6) and elongases (Elovl), where Fads is a major rate-limiting step in the fatty acid biosynthesis pathway (Figure 1-3) (Oboh et al., 2017; Castro et al., 2016). The fatty acyl desaturase gene (*fads*) encodes a protein (Fads) that adds new double bonds to fatty acyl chains, which is essential for the initial step of LC-PUFA biosynthesis. Extreme carnivores, such as felids, have a restricted ability to produce LC-PUFA (Rivers et al., 1976), and so are teleost fish from high trophic levels, which consequently have a dietary requirement for pre-formed LC-PUFA (Li et al., 2010). The vertebrates are having limited capacity to biosynthesis PUFA *de novo*, and different teleost species have a range of capabilities in the bioconversion of dietary

C18 PUFA to LC-PUFA (Sprecher, 2000; Li et al., 2010; Monroig et al., 2011). Among fishes, freshwater fishes have the capacity for bioconversion of dietary C18 PUFA such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) into LC-PUFA, whereas marine fishes have limited ability to biosynthesis the LC-PUFA from LA and ALA substrates (Kanazawa et al., 1979; Li et al., 2010; Monroig et al., 2011). This is due to the lower activity of Fads due to downregulated action of *fads* gene in marine fishes compared to freshwater fishes (Zheng et al., 2009; Castro et al., 2012, 2016). Low expression or absence of *fads* gene is the main reason for the reduced synthesis of LC-PUFA in marine fishes (Li et al., 2010; Castro et al., 2016; Kabeya et al., 2018). FADS have been characterized and well-studied in several species from lower invertebrates to higher vertebrates (Kabeya et al., 2018). Mammals possess two FADS-like enzymes termed FADS1 and FADS2, which are involved in different steps of desaturation activity. FADS1 is a Δ5 desaturase, whereas FADS2 is a Δ6 desaturase with the ability to utilize C18 substrates (Park et al., 2015) to biosynthesis of EFAs, ARA, EPA and DHA. In marine fishes, the biosynthesis of n-3 LC-PUFA is well documented and the process is initiated by the delta 6 fatty acid desaturase (Δ6 Fads), which act on ALA or LA to produce EPA and DHA or ARA through several steps of desaturation, elongation and β-oxidation involved in the FAs biosynthesis (Figure 1-3).

1.5 Dietary sources of EFA

Fish oils are the major source of health-beneficial omega-3 LC-PUFA (Table 1-2). It is well reported that the fish production from marine fisheries has remained static in the next decade and affecting FO production and use in aquaculture (Sargent and Tacon, 1999; Tacon, 2004). Over the past decade, global fish oil production has reached a plateau and is not expected to increase beyond current levels (FAO, 2020). There are numerous lipid sources with a potential use in fish feed as substitutes for FO such as animal by-products, vegetable oils, marine products from lower trophic levels and transgenic plants and the use of ingredients of plant origin as sustainable alternatives to marine oils in fish feed is of great potential. Specifically, plant ingredients have high global availability, as compared to FO, and they have nutritional properties that can largely satisfy the nutritional requirements of the fish.

Table 1-2. Fatty acid composition (% TFA) of fish oil and selected vegetable oil (Source: Turchini et al., 2009)

Fatty acid sources	Saturated FA	MUFA	LA	ALA	ARA	EPA	DHA	n-3 PUFA
Fish oil								
Anchovy	29	25	1.2	0.8	0.1	17	9	31
Vegetable oils								
Soybean oil	15	23	54	8				
Rapeseed oil	4.6	62.3	20.2	0.2	-	-	-	12
Linseed oil	9.4	20.2	13	53	-	-	-	53
Palm oil	48	39	11					

Rapeseed is a bright yellow flowering member of the family Brassicaceae, rich in monounsaturated fatty acid (MUFA) (Table 1-2), being these FAs viewed as a good source to partially replace the fish oil in fish feed. The digestibility of MUFA by fish is higher than that of saturated fats and these fatty acids serve as a good energy source and support physiological requirements (Turchini et al., 2009). Linseed oil (LO) can contain over 50% of total fatty acids as ALA, with ALA:LA ratios usually reported between 3 to 4:1 (Table 1-2). Linseed oil is the best dietary source of ALA fatty acid for fish. This ALA rich LO commonly used in the nutritional programming studies and also for FO replacement studies as LO can be an ideal substrate for EPA and DHA biosynthesis in fish (Turchini et al., 2009; Izquierdo et al., 2015).

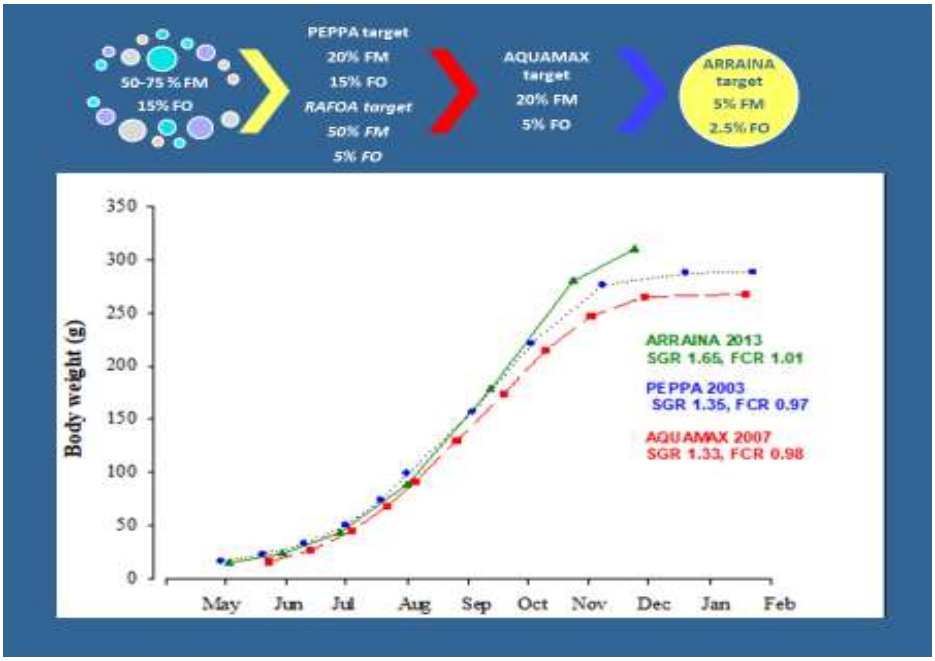

Figure 1-4. Replacement of fish meal and fish oil in diet of gilthead seabream over 15 years of research in European aquaculture (Courtesy of Prof. S. Kaushik).

1.6 Fish oil replacement by vegetable oils

Over the past two decades, considerable research efforts have been put to evaluate the replacement of dietary fish oil by a range of alternative lipid sources, including vegetable oils (e.g., linseed oil, rapeseed oil) and terrestrial animal fats (e.g., beef/pork lard) (Fountoulaki et al., 2009). The vegetable oils are rich in C18-PUFA but devoid of the n-3 LC-PUFA found generally in abundance in fish oil. However, the ability of marine fish to synthesize n-3 and n-6 LC- PUFA, such as EPA, DHA and ARA from the ALA and LA precursors present in vegetable oil is very much limited and hence dietary supply of EPA, DHA and ARA is much essential (Tocher, 2005; Izquierdo, 1996). High levels of vegetable oils in the diets induce changes in the dietary FA profiles, which can affect not only the growth performance but also health status of marine fish (Torrecillas et al., 2017). FM and FO replacement study was undertaken in the European Aquaculture Projects, the FM and FO was replaced from 50-75% and 15% to 5% and 2.5%, respectively (ARRAINA, 2016) (Figure 1-4).

1.7 Limitations in dietary FO replacement by VO on fish reproduction

As described in the above section, broodstock nutrition has long been recognized to have significant effects upon fish gonadal development and egg quality (Watanabe 1984; Izquierdo et al., 2001, 2015). Moreover, among the nutritional constituents of broodstock diets, lipids are the component which mostly affects egg composition (Izquierdo et al., 2001, 2015), and in particular the dietary EFA content (Watanabe et al., 1984). An important function of EPA and DHA is their involvement in reproductive processes, particularly with regard to egg quality and progeny development (Bell et al., 1986, Harel et al., 1994, Fernández-Palacios et al., 1995, Izquierdo et al., 2001, 2015). Indeed, deficiency in the n-3 LC-PUFA content of broodstock diets has been identified as an important factor negatively affecting fish reproductive performances (Izquierdo et al., 2001). Therefore, one of the major consequences when replacing FO by vegetable oil can be related to the changes in EFA dietary profile of broodstock, which in turn may affect the deposition of these fatty acids in ova, resulting in lower quality spawning and eggs and consequently affect offspring development and larval survival. PUFAs are also known to potentially affect reproduction through the regulation of eicosanoids production, particularly prostaglandins, which are involved in several reproductive processes. This includes the production of steroid hormones, gonadal development, and release of sperm and egg. The ARA fatty acid is the main precursor for eicosanoids (PE2) production in fish and the use of VOs, which lack in ARA, could also affect fish reproductive performance (Tocher, 2003; Izquierdo et al., 2001; Bell et al., 1986).

1.8 Nutritional programming approach in fish nutrition

The term "programming" was introduced into scientific literature by Lucas (1991) to describe the process by which exposure to specific stimulus or condition, during critical stages of development, can result in permanent changes in the somatic structures, physiological systems, and metabolic status of the organism. The extension of this concept to the field of early nutrition is known as "nutritional programming" and has been largely studied in mammalian models to understand the consequences in adulthood of an altered nutrition during the pre or post-natal periods (Burdge and Lillycrop, 2010). The mechanisms involved in metabolic regulation by early nutritional events are still not fully understood in many species and it was reported in few species that epigenetic modifications, such as DNA methylation, histone acetylation and non-coding RNAs have been suggested as potential sources for reprogramming the gene expression pattern in mammals (Lillycrop and Burdge, 2012). It was observed that the epigenetic changes are known to be transmitted hereditarily, which largely supports the evidence of vertical transmission of metabolic-related diseases, from mothers to their progeny. Moreover, new data on the role of nutrients, and their availability levels, as modulators of epigenetic mechanisms shows an increasing list of dietary components involved in chromatin remodelling (Badeaux and Shi, 2013).

In fish development, the windows of highest sensitivity and metabolic plasticity are restricted to the periods of embryogenesis and early larval development, similar to mammals. In a study of Senegalese sole (*Solea senegalensis*), muscle growth of larvae undergoing metamorphosis was affected by the early rearing temperature of newly hatched larvae, and it was observed that the lowest rearing temperature (15ºC) induced the methylation of myogenin promoter, which subsequently affected the expression of myogenin gene in the muscle and leads to a reduction in the growth and muscle cellularity (Campos et al., 2013). In the same environmental frame, a research in European seabass upon the shift of sex-ratio in response to temperature showed that exposure to high temperatures (21ºC) increased the methylation level of the gonadal aromatase (*cyp19a*) promoter in both males and females (Navarro-Martín et al., 2011). As result, the expression of aromatase gene was repressed and through the silencing of this gene, the development of males during sex differentiation was promoted. Thus, there are strong evidences that temperature-dependent sex determination in European seabass can be controlled by epigenetic mechanisms of DNA methylation (Navarro-Martín et al., 2011). Several other studies were performed in different fish species to enlighten the role of

environmental factors as modulators of epigenetic mechanisms during the differentiation and determination of sexual phenotypes (Piferrer et al., 2012).

The nutritional stimulus/programming during the early stages of fish life can alter the performance in later stages and this can be achieved through several approaches such as a) maternal nutrition, through nutrient transfer to the yolk reserves and b) the onset of exogenous feeding, when larval nutrition can be manipulated by first-feeds, be it live prey enrichments or inert diets. In GSB, the positive effects of feeding the broodstock with a diet enriched with EFA, were reflected in a better egg quality and larval development (Izquierdo et al., 2015; Turkmen et al., 2020; Xu et al., 2019; Ferosekhan et al., 2020a). Recently, a study in GSB revealed that early nutritional programming could be achieved through parental feeding, with positive long-term metabolic changes of the offspring. When the broodstock was fed with a linseed-oil rich diet (over 60% of fish oil replacement) the resulting progeny proved to be better adapted to use diets with high vegetable oils / vegetable proteins, by showing improved growth rate and feed utilization (Izquierdo et al., 2015). However, even not relevant to the nutritional programming aim, some short-term negative effects were found when FO was replaced 100% by linseed oil in the broodstock diets, with fecundity, spawn quality, growth and Δ6 desaturase gene expression of 45DAH post-larvae negatively affected (Izquierdo et al., 2015).

1.9 Objectives of the studies undertaken

The aim of the present studies undertaken as part of this thesis was to generate novel knowledge on the concept of broodstock nutritional programming in GSB. This thesis intends to study the *fads2* as a potential biomarker of fatty acid status, which can impact the reproductive performance of GSB broodstock. Also, we investigated the correlation between *fads2* gene expression in peripheral blood cells (PBCs) and liver. Besides, another approach of broodstock selection for growth trait and different fatty acids sources on reproductive performance of GSB in mass spawning conditions has been also studied. Therefore, in order to achieve these aims the following specific objectives were addressed,

Broodstock selection based on *fads2* expression

1. To study the fatty acyl desaturase 2 (*fads2*) gene expression correlation between peripheral blood cells (PBCs) and liver to identify a potential biomarker for broodstock selection programme (Chapter 3).

2. To evaluate the broodstock nutritional programming through broodstock selection (high or low *fads2*) and dietary fatty acid profiles on reproductive performance and egg quality in GSB (Chapter 4).

Broodstock selection based on growth trait

3. To study the genetic selection of broodstock for high or low growth trait and fed under high or low n-3 LC-PUFA dietary levels on reproductive performance, steroid hormone levels, sperm, and egg quality of GSB (Chapter 5).

4. To ascertain the effect of high or low growth trait broodstock fed with either fish oil or rapeseed oil based diet on the spawning quality of gilthead seabream, *Sparus aurata* in mass spawning condition (Chapter 6).

CHAPTER 2

GENERAL MATERIALS AND METHODS

Chapter 2

GENERAL MATERIALS AND METHODS

2.1 Ethical statement

All the studies conducted in this thesis were carried out according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at Aquaculture Research Group (GIA), ECOAQUA, University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain. All experiments were performed at the ULPGC, Spain and approved by the Bioethical Committee of the ULPGC and proper care has been taken in handling of live fish in all our studies (REF: 007/2012 CEBA ULPGC).

2.2 Broodstock selection

The general gilthead seabream broodstock selection was as part of ongoing selection programmes (PROGENSA) involving multiple traits and criteria (Navarro et al., 2009; Afonso et al., 2012; Lee-Montero et al., 2013). Specific protocols for broodstock selection are described in detail in each chapter. All the breeders were reared right from the larval stages in the research facilities of the ECOAQUA Institute (ULPGC, Spain) and individually PIT tagged. The breeders were managed by the Aquaculture Research Group (GIA), ULPGC, Spain. Males and females of 2 and 4 year old, respectively, were used in all the studies of this thesis. The broodstock maintenance and other physico-chemical conditions were similar in all the studies. Male maturity was checked by gentle abdominal pressure to determine the spermiation condition and female maturity condition was evaluated by oocyte extraction with a catheter and selection of females bearing only >350 μ diameter oocytes.

2.3 Experimental conditions

During the trials to determine reproductive performance, 2-year old males (around 1 kg) and 4-year old females (around 2 kg) were individually tagged with PIT tags (EID Ibérica SA-TROVAN, Madrid, Spain) and maintained in circular tanks of 1-40 m^3, depending on the objectives of the study. An average ratio female/male biomass of 1.5 was kept in all the experimental tanks. Tanks were supplied with seawater (37 g l^{-1} salinity, 18.5±1.0°C) at a water exchange of 600% daily and maintained under a 12 h photoperiod. Fish were fed twice a day with the selected diet at a feeding rate of 1.25% biomass.

2.4 Relationship between *fads2* gene expression in peripheral blood cells and liver

In Chapter 3, broodstock (n=20) selected and maintained under optimal rearing conditions as described in section 2.2 and 2.3 and fed with experimental diet containing low levels of FM, 5% and FO, 3% were fed at 1% of broodstock body weight twice a day. After a feeding of one month with low FM/FO diet, all the broodstock were fasted overnight and anesthetized with 10 ppm clove oil (clove oil:methanol (50:50) in sea water) to collect peripheral blood and liver samples at the end of the feeding trial. After the feeding trial, blood samples were collected from the caudal vein of the GSB broodfish using sterile syringes and the whole blood samples were transferred to EDTA coated tubes. Whole blood samples were centrifuged at 3000 *g* for 10 min held at (4 °C) to separate blood cells and plasma. The peripheral blood cell samples (PBCs) were stored at –80 °C until RNA extraction. Liver samples were collected from each fish and individually stored in 1.5-ml Eppendorf tube containing RNA Later (Sigma-Aldrich, USA) and was snap frozen in liquid N_2 immediately after sampling. The samples were then stored at –80°C until RNA extraction and analyses.

2.5 Characterization of broodstock with high or low *fads2* expression

In Chapter 4, in order to evaluate the reproductive performance of broodstock with different expression of fatty acid desaturase 2 gene (*fads2*, a key enzyme in synthesis of long chain polyunsaturated fatty acids), 3 months before the spawning season, 185 broodfish were fed twice a day for over one month with a low FM (5%) and FO (3%) diet (Low FM/FO diet), high in LA and ALA (Tables 2-1 and 2-2) and low in LC-PUFA, to induce the up-regulation of *fads2*. After this feeding low FM/FO diet period, blood samples were collected from all the broodfish (n=185) and centrifuged at 3000 *g* for 10 min to separate blood cells and plasma samples. The blood cell samples were stored at -80°C until RNA extraction. Seabream broodfish were divided into two categories, namely high (HD) or low (LD), based on their *fads2* mRNA copy numbers per µl in blood cells. GSB broodfish 18 nos (12 male:6 female) with the highest *fads2* expression values and 18 nos (12 male:6 female) with the lowest ones were selected for the conditioning trial (Phase III). Blood cell *fads2* gene expression level was analysed by droplet digital polymerase chain reaction (ddPCR).

2.6 Characterisation of broodstock with high or low growth trait

In Chapter 5 and 6, to determine the effect of genetic selection for growth trait HG or LG on GSB reproductive performance was performed, the broodstock used in these studies were originated from the third generation of selection under a Spanish National Breeding

Program, the PROGENSA project (Afonso et al., 2012; García-Celdrán et al., 2015; Lee-Montero et al., 2015; Lorenzo-Felipe et al., 2021). Two broodstock groups expressing either high growth (HG) or low growth (LG), selected by BLUP methodology with VCE-v 6.0 software (Neumaier and Groeneveld, 1998), were used for the assessment of reproductive performance in mass spawning. HG and LG traits broodstock were individually marked with PIT tags (EID Iberica SA-TROVAN, Madrid, Spain) and maintained separately for mass spawning in four tanks (10 m^3) at the facilities of ECOAQUA institute (ULPGC, Canary Islands, Spain).

2.7 Experimental diets

2.7.1 Experimental diet 1

The Experimental diet 1 was used in the Phase 1 of Chapters 3 and 4, to induce the up-regulation of *fads2*. In these studies, 3 months before the spawning season, broodfish were fed for one month with a low FM (5%) and FO (3%) diet (Low FM/FO diet), high in LA and ALA (Table 2-1 and 2-2) and low in LC-PUFA to up-regulate the *fads2* in blood or liver.

Table 2-1. Ingredients and proximate composition of the diet used to induce the up-regulation of *fads2* in gilthead seabream broodstock (Phase 1 of Chapters 3 and 4)

Ingredients (%)	**Low FM/FO diet**
Fish meal	5.00
Blood meal (spray-dried)	7.00
Soya protein concentrate	20.00
Corn gluten meal	22.00
Wheat gluten	5.50
Rapeseed meal	11.30
Wheat	6.89
Fish oil	3.00
Rapeseed oil	5.20
Linseed oil	2.60
Palm oil	5.20
Supplemented ingredients	5.49
Vitamin and mineral premix	0.75
Antioxidant	0.05
Yttrium oxide	0.03
Proximate composition	
Crude protein (% dry matter, DM)	45.1
Crude lipid (%DM)	21.7
Moisture (%)	9.0
Ash (%DM)	5.4

Table 2-2. Fatty acid (% TFA) contents of the diet used to induce the up-regulation of *fads2* in gilthead seabream broodstock (Phase 1 of Chapters 3 and 4)

Fatty acid (%TFA)	Low FM/FO
14:0	6.60
14:1n-5	0.10
15:0	0.10
16:0 ISO	0.00
16:0	12.30
16:1n-7	2.10
16:1n-5	0.10
16:2n-4	0.20
17:0	0.30
16:3n-4	0.10
16:3n-1	0.00
16:3n-3	0.00
16:4n-3	0.40
18:0	3.20
18:1n-9	32.30
18:1n-7	2.30
18:1n-5	0.00
18:2n-9	0.00
18:2n-6 (LA)	20.30
18:2n-4	0.10
18:3n-6	0.10
18:3n-4	0.00
18:3n-3 (ALA)	11.80
18:4n-3	0.40
18:4n-1	0.00
20:0	0.40
20:1n-9	1.00
20:1n-7	0.10
20:2n-9	0.00
20:2n-6	0.10
20:3n-9	0.00
20:3n-6	0.00
20:4n-6 (ARA)	0.20
20:3n-3	0.00
20:4n-3	0.10
20:5n-3 (EPA)	2.50
22:1n-11	0.10
22:1n-9	0.30
22:4n-6	0.00
22:5n-6	0.10
22:5n-3	0.30
22:6n-3 (DHA)	1.70
Σ Saturates	22.90
Σ Monoenes	38.40
Σ n-3	17.20

Σ n-6	20.80
Σ n-9	32.6
Σ n-3 LC-PUFA	4.60
DHA/EPA	0.68
DHA/ARA	8.50
n-3/n-6	0.83

2.7.2 Experimental diet 2

2.7.2.1 Broodstock nutritional conditioning diet (FO or RO) used for high (HG) or low (LG) *fads2* expressing gilthead seabream broodstock

As a nutritional conditioning diet for broodstock in the Phase III of Chapter 4, 12 broodstock groups obtained from Phase II were fed one of two different diets (FO or VO diet). The diets were iso-proteic and iso-lipidic, contained either fish oil (FO) or a mixture of 20% fish oil (FO) and 80% rapeseed oil (RO) and were produced by Skretting ARC (Stavanger, Norway) (Tables 2-3). Compared to the FO diet, the RO diet had higher levels of LA and ALA, and reduced levels of saturated, monoenoic and EPA and DHA. (Table 2-4).

Table 2-3. Ingredients and proximate composition of experimental diets (FO and RO) used for nutritional conditioning in Phase III of Chapter 4

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

Table 2-4. Fatty acid (% TFA) composition of experimental diets (FO and RO) used for nutritional conditioning in Phase III of Chapter 4

Fatty acid (%TFA)	**FO diet**	**RO diet**
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 (LA)	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 (ALA)	1.30	4.95
18:4n-3	2.19	1.22
18:4n-1	0.00	0.11
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 (ARA)	1.04	0.43
20:3n-3	0.15	0.12
20:4n-3	0.57	0.35
20:5n-3 (EPA)	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 (DHA)	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

2.7.3 Experimental Diet 3

2.7.3.1 Broodstock nutritional conditioning diet for high (HG) or low (LG) growth trait gilthead seabream broodstock

In Phase II of Chapter 5, the broodstock diets used for nutritional conditioning were adapted for a higher content in ALA, a precursor of n-3 LC-PUFA, in comparison to previous trials. These experimental diets were formulated to be iso-proteic and iso-lipidic with either fish oil (FO diet) or a blend of vegetable oils (VO diet, containing rapeseed and linseed oil) as the lipid source and were produced by Skretting ARC, Stavanger, Norway (Table 2-5). The VO diet had higher levels of LA and ALA fatty acids and reduced levels of saturated, monoenoic and EPA and DHA (Table 2-6).

Table 2-5. Ingredients and proximate composition of the broodstock diets (FO or VO) used for high (HG) or low (LG) growth trait gilthead seabream broodstock in Phase II of Chapter 5

Feed ingredients (%)	FO diet	VO diet
Fish meal, North-Atlantic	57.33	57.33
Krill meal	7.00	7.00
Squid meal	3.00	3.00
Wheat	21.99	21.99
Fish oil, South American	9.96	0.00
Rapeseed oil	0.00	8.45
Linseed oil	0.00	1.50
Vitamin-Mineral premix*	0.50	0.50
Astaxanthin 10%	0.03	0.03
L-Histidine HCl	0.20	0.20
Proximate composition		
Crude protein (% dry matter, DM)	51.7	51.2
Crude lipid (% DM)	16.8	16.5
Ash (% DM)	10.6	10.4
Moisture (%)	8.4	8.9

Table 2-6. Fatty acid (% TFA) composition of the broodstock diets (FO or VO) used for high (HG) or low (LG) growth trait gilthead seabream broodstock in Phase II of Chapter 5

Fatty acid (%TFA)	FO diet	VO diet
14:0	3.44	2.17
14:1n-7	0.03	0.03
14:1n-5	0.11	0.08
15:0	0.30	0.16
15:1n-5	0.02	0.03
16:0 ISO	0.06	0.03
16:0	13.29	9.18
16:1n-7	5.12	2.38
16:1n-5	0.22	0.09

16:2n-6	0.01	0.00
16:2n-4	0.61	0.19
17:0	0.67	0.10
16:3n-4	0.13	0.15
16:3n-3	0.13	0.07
16:3n-1	0.08	0.05
16:4n-3	0.93	0.28
16:4n-1	0.03	0.02
18:0	3.54	2.53
18:1n-9	12.72	35.07
18:1n-7	2.93	2.79
18.1n-5	0.18	0.12
18:2n-9	0.04	0.02
18.2n-6	4.56	13.49
18:2n-4	0.23	0.05
18:3n-6	0.27	0.00
18:3n-4	0.15	0.07
18:3n-3	1.59	9.58
18.3n-1	0.03	0.01
18:4n-3	2.14	1.01
18:4n-1	0.14	0.03
20:0	0.52	0.49
20:1n-9	0.47	0.24
20:1n-7	3.88	3.19
20.1n-5	0.34	0.12
20:2n-9	0.05	0.02
20:2n-6	0.23	0.14
20:3n-9	0.09	0.04
20:3n-6	0.12	0.04
20:4n-6	1.00	0.28
20:3n-3	0.12	0.06
20:4n-3	0.67	0.21
20:5n-3	14.97	4.81
22:1n-11	5.20	3.20
22:1n-9	0.73	0.64
22:4n-6	0.14	0.06
22:5n-6	0.33	0.09
22:5n-3	1.91	0.36
22:6n-3	15.56	6.23
Total saturates	21.75	14.63
Total monoenes	31.93	47.98
Total n-3	38.01	22.62
Total n-6	6.659	14.10
Total n-9	14.09	36.03
Sum n-3 LC-PUFA	33.23	11.67
EPA/ARA	15.02	17.41
ARA/EPA	0.067	0.057
DHA/EPA	1.039	1.296
DHA/ARA	15.62	22.57
n-3/n-6	5.71	1.60

2.7.4. Experimental Diet 4

2.7.4.1 Broodstock nutritional conditioning diet for high (HG) or low (LG) growth trait gilthead seabream broodstock

In Phase II of Chapter 6, the experimental broodstock feeds were formulated to be iso-proteic and iso-lipidic with either fish oil (FO diet) or a rapeseed oil (RO) as the lipid source and were produced by Skretting ARC, Stavanger, Norway (Tables 2-7 and 2-8). As like other experiments, in this study also RO diet was formulated to contain higher levels of LA and ALA fatty acids as a precursors for LC-PUFA biosynthesis and reduced levels of saturated, monoenoic, and EPA and DHA fatty acids (Table 2-8).

Table 2-7. Ingredients and proximate composition of the broodstock experimental diets used for mass spawning study in Phase II of Chapter 6.

Feed ingredients (%)	Diet FO	Diet RO
Fish meal (North-Atlantic 12C)	59.36	59.36
Krill meal	7.00	7.00
Squid meal	3.00	3.00
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 (% dry matter, DM)	58.9	58.1
Crude lipid (% DM)	21.3	22.1
Ash (% DM)	9.8	9.8

Table 2-8. Fatty acid profiles (% total fatty acids) of FO and RO broodstock experimental diets in Phase II of Chapter 6.

%TFA	FO diet	RO diet
14:0	6.01	2.22
14:1n-7	0.03	0.01
14:1n-5	0.20	0.07
15:0	0.45	0.18
15:1n-5	0.03	0.01
16:0 ISO	0.08	0.03
16:0	17.65	9.42
16:1n-7	6.93	2.20
16:1n-5	0.21	0.07
16:2n-6	0.02	0.01
16:2n-4	0.85	0.24
17:0	0.99	0.23

16:3n-4	0.14	0.10
16:3n-3	0.16	0.07
16:3n-1	0.07	0.03
16:4n-3	1.08	0.33
18:0	3.45	2.41
18:1n-9	9.96	36.94
18:1n-7	2.70	2.74
18.1n-5	0.20	0.14
18:2n-9	0.10	0.02
18.2n-6	2.64	12.32
18:2n-4	0.26	0.08
18:3n-6	0.24	0.08
18:3n-4	0.26	0.10
18:3n-3	1.12	4.85
18.3n-1	0.05	0.02
18:4n-3	2.70	1.30
18:4n-1	0.20	0.07
20:0	0.29	0.48
20:1n-9	0.30	0.23
20:1n-7	3.70	3.81
20:1n-5	0.27	0.12
20:2n-9	0.12	0.02
20:2n-6	0.20	0.14
20:3n-9	0.07	0.03
20:3n-6	0.13	0.04
20:4n-6	0.94	0.35
20:3n-3	0.13	0.08
20:4n-3	0.82	0.29
20:5n-3	13.47	5.26
22:1n-11	4.37	3.92
22:1n-9	0.43	0.78
22:4n-6	0.13	0.05
22:5n-6	0.32	0.13
22:5n-3	1.83	0.52
22:6n-3	13.71	7.46
Total saturates	28.84	14.94
Total monoenes	29.32	51.04
Total n-3	35.02	20.16
Total n-6	4.61	13.11
Total n-9	10.90	37.99
Sum n-3 LC-PUFA	29.96	13.61
EPA/ARA	14.39	15.08
DHA/EPA	1.02	1.42
DHA/ARA	14.65	21.39
n-3/n-6	7.60	1.54

2.8 Experimental samplings

2.8.1 Broodstock

2.8.1.1 Plasma sex steroid hormones (Chapter 4 and 5)

All the breeders were fasted overnight and anesthetized with clove oil (10 ppm clove oil : methanol (50:50) in sea water) to collect blood samples. Blood was taken from the caudal vein using sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 3.0 mL K3-EDTA tubes (L.P. Italiana, Milan, Italy). Whole blood samples were centrifuged at 3000 g for 10 min at 4 °C and plasma was separated and stored at -80 °C for sex steroid hormone analyses. Plasma sex steroid hormones were measured by enzyme immunoassays (EIA) as described for European seabass for male testosterone (T) (Rodríguez et al., 2000), 11-ketotestosterone (11-KT) (Rodríguez et al., 2001) and female 17β-estradiol (E2) (Molés et al., 2008). Plasma steroids were extracted with methanol and supernatants were dried and reconstituted in EIA buffer (potassium phosphate 0.1 M, pH 7.4 containing 0.01% sodium azide, 0.4 M NaCl, 0.001 M EDTA and 0.1% BSA). The assays were performed in 96-well plates coated with mouse anti-rabbit IgG monoclonal antibodies (Sigma-Aldrich, R-1008). Steroid standard curves (ranging from 0.0024-5.0 ng/ml for T; 0.0005-1.0 ng/ml for 11-KT and 0.039-80.0 ng/ml for E2; Sigma-Aldrich) or plasma samples were run in duplicate and added to the wells together with the corresponding acetylcholinesterase (AChE) tracer: (T-AchE, 11-KT-AChE or E2-AChE; Cayman Chemical, Michigan, USA) and rabbit antiserum (anti-T, anti-11-KT or anti-E2), and incubated at 37ºC (E2) or 4ºC (T and 11-KT). Next, plates were rinsed, and colour development was performed by addition of Ellman reagent. Optical density was read at 405 nm using a microplate reader (Bio-Rad 3550). The sensitivities of the assays (80% of binding) were 0.025 ng/ml for T, 0.0049 ng/ml for 11-KT and 0.30 ng/ml for E2. The inter-assay coefficients of variation at 50% of binding were 0.42% with a 0.90 slope for T, 32.6% with a 0.84 slope for 11-KT and 2.05% with a 0.83 slope for E2. The intra-assay coefficients of variation were 2.93% (n=4) for T, 5.65% (n = 6) for 11-KT and 0.83% (n=4) for E2.

2.8.1.2 Sperm quality (Chapter 4 and 5)

For sperm collection, fish were anesthetized as mentioned above and sperm was collected from the blot dried genital pore after a gentle abdominal massage to induce spermiation and taking care to avoid contamination with mucus, water, faeces, or urine. The collected sperm was stored on ice until transferred to a 4 °C refrigerator. The sperm quality

parameters that were evaluated included sperm concentration (number of spermatozoa/ml sperm, 10^9 ml^{-1}), spermatocrit %, sperm motility % (percentage of spermatozoa showing forward motility) and sperm motility duration (min). Sperm concentration was estimated after a 1000-fold dilution with sperm inactivation media using a Neubauer haematocytometer under 400x magnification. Sperm motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 µl of sperm with 50 µl of seawater (Cabrita et al., 2005; Felip et al., 2009).

2.8.1.3 Evaluation of egg and larval quality (Chapter 4, 5 and 6)

Spawning quality was determined before and after feeding the experimental diets for each experiment. The spawning quality parameters were determined using eggs, which were collected daily from each tank at around 08:00 h and concentrated in 10 L containers. Immediately, eggs were transferred to the laboratory and aeration supplied to each to ensure aeration of the eggs through the water column. A 5 or 10 mL sample (n=3) was taken by using graduated glass pipet and transferred to a Bogorov chamber. Eggs were counted and observed under a binocular microscope (Leica Microsystems, Wetzlar, Germany) in five replicates to determine: the total number of eggs, the percentage of fertilized eggs and the egg morphological characteristics. Egg viability rate was calculated as the percentage of morphologically normal eggs at the morula stage, described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios et al., 2011). After checking egg viability, viable eggs were randomly taken and transferred to 96 well microplates using a micropipette (0.7 mL of filtered and sterilized seawater and one egg per well). Microplates were observed under a microscope to ensure that there is a single fertilized egg at each well. These eggs were kept in temperature-controlled at a constant temperature of 19-23 °C as per the recorded water temperature. After 24 and 72 h of incubation, microplates were observed under a microscope to calculate and count the hatching rate and survival rate after 3 (days post hatch). With these percentage values, the total number of fertilized, viable and hatched eggs and larvae survived after 3 dph were calculated per spawn/kg female.

2.9 Chemical analysis (Chapter 3, 4, 5 and 6)

2.9.1 Moisture

Moisture content was determined by oven drying to constant weight at 110 °C, with a first 24 h drying period, followed by 1 h periods until weight was not reduced any further or in case of higher weight observed from the weight obtained previously. Sample weight

(between 200 - 500 mg, depending on sample size from different tissues) was recorded before drying and after each drying period, following the cooling in a desiccator for 10 min. Moisture was expressed as a percentage of the weight according to Official Methods of Analysis (AOAC, 1995).

2.9.2 Ash

Ash content was determined by drying the samples (between 200 - 500 mg, depending on sample size from different tissues) in a muffle furnace at 450°C until a constant weight was attained (AOAC, 1995).

2.9.3 Crude protein

Proteins were estimated from analysis of the total nitrogen present in the sample, using the Kjeldhal method (AOAC, 1995). Briefly, after the digestion of the sample (between 200-500 mg, depending on sample size from different tissues) with concentrated sulphuric acid at a temperature of 400°C, the total nitrogen content was determined and converted to total crude protein value by multiplying by the empirical factor 6.25.

2.9.4 Crude lipid

Lipids were extracted following the method of Folch et al. (1957). The method starts taking a sample amount between 50-200 mg and homogenizing it in an Ultra-Turrax (IKA-Werke, T25 BASIC, Staufen Germany,) during 5 min in a solution of 5 mL of chloroform: methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric pressure through glass wool and 0.88% KCI added to increase the water phase polarity. After decantation and centrifugation at 2000 rpm 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 weighed. Neutral and polar fractions were separated by adsorption chromatography on silica cartridges Sep-pak (Waters, Milford, MA) as described by (Juaneda and Rocquelin, 1985).

2.9.5 Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipids with 1% sulphuric acid in methanol (Christie, 1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50 °C. Afterwards, FAMES were extracted with hexane:diethyI ether (1:1, v/v) and purified by adsorption chromatography on NH Sep-pack cartridges (Waters S.A., Massachussets, USA) + (Christie, 1982). FAMES were separated by Gas-Liquid Chromatography (GLC) (Agilent 7820A, CA, USA) in a SupercoIvax-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 by Izquierdo et al. (1990). 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).

2.10 Molecular studies (Chapter 3, 4, and 5)

Molecular studies in all the studies undertaken as part of this thesis were mainly focused to identify expression differences in selected genes of PUFA metabolism and stress resistance. Three different approaches were used to examine the effects of broodstock diets on the progeny. These were real-time polymerase chain reaction (PCR) analysis for experiment 1,2,3 to identify the expressions as fold-change to a control group, digital droplet PCR (ddPCR) for experiments 3 and 4 to measure the absolute gene expression and micro-array for a global view of the lipid metabolism related genes of gilthead sea bream. In the last studies, experiments 3 and 4, with the genome sequence of gilthead sea bream became available which has given more opportunities to analyze different epigenetic mechanisms such as DNA promoter methylation of the genes investigated. Primers used in these studies were presented in each chapter.

2.10.1 RNA extraction

Tissue samples were taken and preserved directly in RNA Later (Sigma-Aldrich) overnight at 4°C. Then RNA Later was removed, and samples kept at -80°C until RNA extraction and analyses. Digestion of tissues for RNA extractions was done using TRI Reagent (Sigma-Aldrich, Missouri, USA). For the digestion, approximately 100 mg of tissue were weighted, 1 mL of TRI reagent and four pieces of 1 mm diameter zirconium glass beads, were added to 2 mL volume of Eppendorf tubes. Samples were homogenized using Tissue Lyzer-II (Qiagen, Hilden, Germany) for 60 seconds with a frequency of 30s until tissue was completely dissolved. 250 µL chloroform was added to homogenized samples and then centrifuged at 12000 *g* for 15 min at 4 °C for phase separation. The transparent upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane. After that RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen) with the protocol supplied by the manufacturer.

2.10.2 Digital droplet PCR analysis for the absolute gene expression

Tissue RNA extraction was made using the protocol explained above for the real time PCR. A modified version of the RNA extraction method was used for the blood. The whole

blood was taken from the caudal vein of the brood fish for the selection of the individuals. Prior to measurements, all fish were anesthetized with 10 ppm clove oil/methanol (50:50) in sea water. 2.5 mL blood was taken with 2.5 mL sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 2.5 mL K 3 EDTA tubes (L.P. Italiana, Milan, Italy), then 500 µL transferred to 2 mL Eppendorf tubes. Whole blood samples were kept on ice during the sampling and were immediately centrifuged at 10000 rpm, 4 °C for 20 minutes. Plasma samples were separated, and erythrocytes were snap frozen with liquid nitrogen and kept at -80 °C until RNA extraction. Samples were kept at room temperature until samples were thawed completely. RNA was extracted as per the procedure described in section 2.10.1 and RNA integrity was assessed in agarose gel as per standard procedure.

2.11 Statistical analysis

Data are reported as mean ± standard deviation. Data were compared statistically using the analysis of variance (ANOVA), at a significance level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene's tests, respectively. Otherwise, an arcsine transformation was performed to attain normality. When arcsine transformed data were not normally distributed, then Kruskal–Wallis non-parametric test was applied to the non-transformed data. An independent sample student's *t* test was performed to compare egg biochemical and fatty acid composition to check the broodstock selection (HD or LD and HG or LG) effect and also broodstock for body weight, Hepato somatic index (HSI%), gonado somatic index (GSI%), PBCs, liver *fads2* expression and hepatic fatty acid composition. One way and two-way ANOVA were applied to the results of sperm and egg and larval quality parameters (total eggs; fertilized eggs; viable eggs; hatched larvae; 3dph larvae per spawn per kg female and fertilization, egg viability, hatching and larval survival rates), egg biochemical and fatty acid composition to determine the combined effects of broodstock selection (HD or LD and HG or LG) and diet (FO or VO and FO or RO). Linear regression analysis was performed for relationships between specific fatty acids contents in eggs (n-3 LC-PUFA) and egg viability or larval survival (3dph) %. Pearson's correlation coefficient was calculated for liver *fads2* expression on hepatic fatty acid of either male or female broodstock and also for to check the relationship between spawning quality parameters. All data were analysed using the program IBM SPSS version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA).

CHAPTER 3

Impact factor: 3.817

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

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Life **2020**, *10*(7), 117; https://doi.org/10.3390/life10070117

Chapter 3

Abstract

The principle aim of this study is to elucidate the relationship between fatty acid desaturase 2 gene (*fads2*) expression pattern in peripheral blood cells (PBCs) and liver of gilthead seabream (GSB), *Sparus aurata,* broodstock in order to determine the possible use of *fads2* expression as a potential biomarker for the selection of broodstock. This selection could be utilized for breeding programs aiming to improve reproduction, health, and nutritional status. PIT tagged GSB broodstock (Male - 1.22 ± 0.20 kg; 44.8 ± 2 cm and female – 2.36 ± 0.64 kg; 55.1 cm) were fed a diet containing low levels of fish meal and fish oil (EPA 2.5; DHA 1.7 and n-3 LC-PUFA 4.6 % TFA) for one month. After the feeding period, *fads2* expression in PBCs and liver of both male and female broodstock were highly significantly correlated (r=0.89; $P<0.001$). Additionally, in male broodstock, liver *fads2* expression was significantly correlated ($P<0.05$) to liver contents in 16:0 (r=0.95; $P=0.04$) and total saturates (r=0.97; $P=0.03$) as well as to 20:3n–6/20:2n–6 (r=0.98; $P=0.02$) a Fads2 product/precursor ratio. Overall, we found a positive and significant correlation between *fads2* expression levels in the PBCs and liver of GSB broodstock. PBCs *fads2* expression levels indicate a strong potential for utilization as a non-invasive method to select animals having increased fatty acid bioconversion capability, better able to deal with diets free of fish meal and fish oil.

Keywords: biomarker; broodstock; fatty acyl desaturase (*fads2*); fatty acid; lipid metabolism

3.1 Introduction

The ability of teleost to biosynthesize long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA), such as arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3), from 18 carbon PUFA substrate, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) is limited (Monroig et al., 2010; Monroig et al., 2011; Oboh et al., 2017). Knowledge on this is of vital importance in order to quantify the dietary requirements for essential fatty acids (EFA) in farmed fish and to tailor the fatty acid composition of the fish fillet for human consumption (Tocher, 2015; Tocher et al., 2019). The fatty acid biosynthesis comprises of several steps catalyzed by fatty acyl desaturases (Fads1-Δ5 and Fads2-Δ6 desaturase) and elongation of very long chain fatty acids proteins (Elovl), where Δ6 desaturase is a major rate-limiting step in the fatty acid biosynthesis pathway (Figure 1-3) (Castro et al., 2016; Oboh et al., 2017). The Δ6 desaturase introduce new double bonds into fatty acyl chains, essential to the first step in the LC-PUFA biosynthesis and is encoded by the fatty acyl desaturase gene (*fads*). Carnivorous species such as felids have very limited ability to synthesize LC-PUFA (Rivers et al., 1976), as well as teleost fish from higher trophic levels, which consequently have a dietary requirement for pre-formed LC-PUFA (Li et al., 2010). In general, the majority of the vertebrates lack the capacity to biosynthesize PUFA de novo, and different teleost species have a wide range of bioconversion capability for converting dietary C18 PUFA to LC-PUFA (Sprecher, 2000; Li et al., 2010; Monroig et al., 2011). Among teleost, freshwater fish and especially fish from lower trophic levels are considered capable of bioconversion of dietary C18 PUFA such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) into LC-PUFA, whereas marine teleost have limited ability to biosynthesize the LC-PUFA from LA and ALA substrates (Kanazawa et al., 1979; Li et al., 2010; Monroig et al., 2011). This is imputed to the lower activity of Fads, which is due to the lower expression of the *fads* gene in marine fish compared to freshwater fish (Zheng et al., 2009; Castro et al., 2012; Geay et al., 2012; Castro et al., 2016). Low expression or absence of fads gene is the main reason for the reduced synthesis of LC-PUFA in marine fish (Li et al., 2010; Castro et al., 2016; Kabeya et al., 2018). Fads has been characterized and studied in several organisms from lower invertebrates to higher vertebrates (Kabeya et al., 2018). Mammals possess two FADS enzymes termed FADS1 and FADS2, which are involved in different steps of desaturation activity. FADS1 is a Δ5 desaturase, whereas FADS2 is a Δ6 desaturase with the ability to utilize C18 substrates (Park et al., 2015) in order to biosynthesize ARA, EPA and DHA. In marine fish, it is well documented that the biosynthesise of n-3 LC-PUFA is initiated

by the delta 6 fatty acid desaturase (Δ6 Fads), which acts on ALA or LA to produce EPA and DHA or ARA through several steps of desaturation, elongation and β-oxidation (Figure 1-3).

Fads2 from most of the teleost species show Δ6 desaturase activity, its activity was also identified in many species such as zebra fish, *Danio rerio* (Hastings et al., 2001), gilthead seabream (GSB) (*Sparus aurata*) (Seiliez et al., 2003; Zheng et al., 2004; Izquierdo et al., 2008; Monroig et al., 2011; Vagner and Santigosa, 2011), European seabass (*Dicentrarchus labrax*) (González-Rovira et al., 2009; Santigosa et al., 2011), black seabream (*Acanthopagrus schlegeli*) (Kim et al., 2011), meagre (*Argyrosomus regius*) (Monroig et a*l.*, 2013), orange-spotted grouper (*Epinephelus coioides*) (Li et al., 2014), and nibe croaker (*Nibea mitsukurii*) (Kabeya et al., 2015), rabbit fish (*Siganus canaliculatus*) (Lim et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Zheng et al., 2004), Atlantic salmon (*Salmo salar*) (Zheng et al., 2005; Monroig et al., 2010) and Atlantic cod (*Gadus morhua*) (Tocher et al., 2006). Analysis of the different studies on enzyme activities of several species shows that many species have several desaturase activities. For example, zebrafish Δ6 desaturase, which was the first functionally characterized Fads2 in a teleost, showed three Δ5, Δ6 and Δ8 desaturase activities (Hastings et al., 2001). Similarly, dual Δ6 and Δ8 desaturase activity has been reported in other teleost Fads2 including GSB (Zheng et al., 2004; Monroig et al., 2011), rabbitfish (Li et al., 2010; Monroig et al., 2011; Lim et al., 2014), rainbow trout (Zheng et al., 2004), turbot (Zheng et al., 2004), Atlantic salmon (Monroig et al., 2010; Monroig et al., 2011) and grouper (Li et al., 2014). The *fads2* gene expression in different tissues has been studied in several marine fish species although the importance and implication of *fads2* gene in fish growth and reproduction is has not been deeply studied except for a few studies by our own research group (Izquierdo et al., 2015; Turkmen et al., 2017b; Turkmen et al., 2020).

Lipid metabolism and fatty acid synthesis occurs mainly in the liver, then fatty acids are transported through the blood to the rest of the tissues in the body for utilization in diverse physiological functions (Tocher, 2003). Knowing the activity and expression of Δ6 desaturase and *fads2* in liver is paramount in order to ascertain the FAs synthesis and mobilization to various tissues. The measurement of *fads2* expression in the liver however requires sacrificing the animal and it is a major constraint for working on live fish. Hence, it is necessary to develop a non-invasive method to study the *fads2* expression in fish. It is well known that blood is the most accessible tissue to study the well-being of an animal to predict or assess the physiological conditions of other tissues by means of a non-invasive manner. In humans, peripheral blood

cells (PBCs) are widely used to check the health status by analyzing the gene expression pattern in peripheral blood and to predict the same gene expression in non-accessible tissues like brain, liver, gonad, kidney, lungs etc. In various human studies, expression of many health status indicative genes in PBCs show significant correlations to the respective gene expression in different other organ tissues (Liew et al., 2006; Halloran et al., 2015; Palmer et al., 2019; Hasan et al., 2020).

The presence of a nucleus and functional mitochondria in red blood cells of fish makes blood a promising tissue to analyze the gene expression and metabolic responses in an integrative and non-invasive manner. Some studies with GSB have addressed the issue to determine whether samples collected without sacrificing animals provide a reliable measure of mitochondrial functioning and energy metabolism at the level of the whole organism (Martos-Sitcha et al., 2019). In one such study using transcriptome analysis, it was observed that whole blood cells gene expression of hypoxia induced GSB juveniles reflected the metabolic condition and mitochondrial respiration of target tissues (Martos-Sitcha et al., 2019). Such an approach could be used in GSB broodstock to understand the relationship between *fads2* expression in PBCs and liver to identify the potential of using *fads2* as a biomarker to assess the role of n-3 LC PUFA in reproductive performance. The correlation between PBCs and hepatic *fads2* expression has not been studied so far in any fish or even other animals. However, the relative *fads2* expression between various tissues has been studied and reported in humans (Reynolds et al., 2018) as well as in some fish such as the Japanese seabass (*Lateolabrax japonicus*) or the golden pompano (*Trachinotus ovatus*) (Xu et al., 2014; Zhu et al., 2019). In Japanese seabass, *fads2* expression was found to be higher in brain, eyes, liver and intestine than in kidney, skin, muscle, gill, spleen, stomach, blood, and heart (Xu et al., 2014). In golden pompano, the *fads2* expression level was higher in brain, in comparison to the small intestine and the female gonads, whereas lower expression levels were observed in fin, gill, blood, and kidney (Zhu et al., 2019).

To our knowledge, there is no known information on the comparative relationship between blood and liver *fads2* expression patterns, nor potential differences in male and female fish. The blood *fads2* gene expression as a biomarker could be useful to study not only the fatty acid metabolism in fish but also to assess the nutritional quality of fish fillet (Turkmen et al., 2017b; Turkmen et al., 2019,2020) or the reproductive performance in fish (Turkmen et al., 2020). The *fads2* expression pattern in animals are species and sex specific (Bakewell et al., 2006; Childs

et al., 2010a; Childs et al., 2010b; Childs et al., 2012). In an earlier study, we observed that *fads2* expression in the blood of GSB broodstock was higher in female than in male (Turkmen et al., 2020) and the GSB exhibit great variations in the *fads2* expression among individuals. Therefore, the present study was undertaken to relate the expression pattern of PBCs and hepatic *fads2* in fish to elucidate the relationship in GSB broodstock to identify the potential of selecting PBCs *fads2* as a biomarker to understand the reproductive performance of fish. A second objective was to analyze the hepatic fatty acid profile of GSB broodstock fish exhibiting different *fads2* expression levels in peripheral blood cells and liver.

3.2 Materials and Methods

3.2.1 Broodstock management and feeding

Gilthead seabream broodstock were individually PIT tagged (EID Iberica SA-TROVAN, Madrid, Spain) and maintained in a 10 tons square tank. Male (1.22 ± 0.20 kg; 44.8 ± 2 cm; 4 fish) and female (2.36 ± 0.64 kg; 55.1 ± 2 cm; 16 fish) broodstock were maintained in the same tank. Both male and female broodstock were studied for their *fads2* expression pattern in blood cells and liver. The tank was supplied with seawater (37 g L^{-1} salinity, 17.0-20.0 °C) at a daily water exchange rate of 600% and fish were maintained under natural photoperiod. All the broodstock were fed 1% body weight twice a day for one month with a diet containing low levels of fish meal (FM, 5%) and fish oil (FO, 3%) (Table 3-1). The experimental diet was produced by Biomar (Torrecillas et al., 2017) and re-pelletized at the feed manufacturing facilities of GIA (ECOAQUA Institute, ULPGC, Spain). The diet (Table 3-2) was formulated to contain high levels of vegetable oils and thus contained high levels of oleic acid (18:1n-9) (32.3 % of total fatty acids, TFA), LA (18:2n-6) (20.3 %TFA) and ALA (18:3n-3) (11.8 %TFA) while containing low concentrations of EPA (2.5 %TFA), DHA (1.7 %TFA) and n-3 LC-PUFA (4.6 %TFA) in order to up-regulate the fatty acid desaturase 2 (*fads2*) gene expression (Torrecillas et al., 2017).

Table 3-1. Ingredients (%) and proximate composition of the low fish meal and low fish oil diet fed to gilthead seabream broodstock

Ingredients (%)	**Experimental diet**
Fish meal (South American)	5.00
Blood meal (spray-dried)	7.00
Soya protein concentrate	20.00
Corn gluten meal	22.00
Wheat gluten	5.50
Rapeseed meal	11.30
Wheat	6.89
Fish oil (South American)	3.00
Linseed oil	2.60
Palm oil	5.20
Rapeseed oil	5.20
Supplemented ingredients[1]	5.49
Vitamin and mineral premix[2]	0.75
Antioxidant-Ethoxyquin	0.05
Yttrium oxide	0.03
Proximate composition	
Crude protein (% dry matter, DM)	45.1
Crude lipid (% DM)	21.7
Ash (% DM)	5.4
Moisture (%)	9.0

[1] Supplemented ingredient contain - lysine, methionine, monocalcium phosphate, choline, inositol, phospholipids. [2] Vitamin and 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.

Table 3-2. Fatty acids profiles of low fish meal and fish oil diet for gilthead seabream broodstock (% total fatty acids)

Fatty acid (%TFA)	Experimental diet
14:0	6.6
14:1n-5	0.1
15:0	0.1
16:0	12.3
16:1n-7	2.1
16:1n-5	0.1
16:2n-4	0.2
17:0	0.3
16:3n-4	0.1
16:4n-3	0.4
18:0	3.2
18:1n-9	32.3
18:1n-7	2.3
18:2n-6 (LA)	20.3
18:2n-4	0.1
18:3n-6	0.1
18:3n-3 (ALA)	11.8
18:4n-3	0.4
20:0	0.4
20:1n-9	1.0
20:1n-7	0.1
20:2n-6	0.1
20:4n-6 (ARA)	0.2
20:4n-3	0.1
20:5n-3 (EPA)	2.5
22:1n-11	0.1
22:1n-9	0.3
22:5n-6	0.1
22:5n-3 (DPA)	0.3

22:6n-3 (DHA)	1.7
Total saturates	22.9
Total monoenes	38.4
Total n-3	17.2
Total n-6	20.8
Total n-9	32.6
Total n-3 LC-PUFA	4.6

3.2.2 Blood and liver sample collection and storage

All broodfish (n=20) were fasted overnight and anesthetized with 10 ppm clove oil (clove oil:methanol (50:50) in sea water) to collect peripheral blood and liver samples at the end of the feeding trial. After the feeding trial, blood samples were collected from the caudal vein of the GSB broodfish using sterile syringes and the whole blood samples were transferred to EDTA coated tubes. Whole blood samples were centrifuged at 3000*g* for 10 min held at (4 °C) to separate blood cells and plasma. The peripheral blood cell samples (PBCs) were stored at –80 °C until RNA extraction. Liver samples were collected from each fish and individually stored in 1.5-ml Eppendorf tube containing RNA Later (Sigma-Aldrich, USA) and was snap frozen in liquid N_2 immediately after sampling. The samples were then stored at –80°C until RNA extraction and analyses.

3.2.3 Molecular study – RNA extraction and cDNA synthesis

Total RNA from PBCs (300-400 µl) and liver samples (60-70 mg) were extracted following the manufacturer's protocol using the RNeasy Mini Kit (Qiagen) and both blood cells and liver samples were completely homogenized with glass beads using the Tissue Lyzer-II (Qiagen, Hilden, Germany) with TRI Reagent (Sigma-Aldrich, USA). Chloroform was added to the homogenized samples and centrifuged at 12,000 *g*, 15 min, 4 °C for clear phase separation. The clear upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into the RNeasy spin column, where total RNA bound to a membrane and RW1 (700 µl) and RPE (500 µl) buffers (Qiagen) were used to wash away contaminants. Total RNA from RNeasy spin column was eluted with 50 µL of RNase-free water. The extracted total RNA quality and quantity were checked using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). We used 100 ng RNA for the cDNA synthesis, and it was carried out as following the standard protocol and cDNA synthesis was performed using the iScript cDNA

Synthesis Kit (Bio-Rad) according to manufacturer's instructions in the iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The efficiency of Δ6 desaturase primer was tested with serial dilutions of a cDNA pool (1, 1:10, 1:100, 1:200 and 1:1000). Primers for GSB Δ6 desaturase were redesigned with reference to publications in NCBI (National Center for Biotechnology Information) as follows (Izquierdo et al., 2015; Turkmen et al., 2017b; Turkmen et al., 2019, 2020):

Gene – *fads2* (Δ6 desaturase; GenBank: GQ162822.1)

Forward primer sequence (5′ → 3′) GCAGAGCCACAGCAGCAGGGA

Reverse sequence (3′ → 5′) CGGCCTGCGCCTGAGCAGTT

3.2.4 Digital droplet PCR (ddPCR) analysis for absolute gene expression

Total RNA extraction from peripheral blood cells and liver were made using a similar protocol described above for the qPCR. Absolute gene expression of PBCs and liver *fads2* analysis were performed using Digital Droplet PCR (ddPCR) (Bio-rad QX200, Hercules, California, USA) systems, by using cDNA obtained as mentioned in Section 2.3. Sample preparation for ddPCR was carried out as per the manufacturer's protocol. The master mixes for *fads2* gene were prepared including 10 µL EvaGreen super mix (Bio-rad, Hercules, California, U.S.A.), 0.2 µL F primer (10 pmol/ µL), 0.2 µL R primer (10 pmol/ µL), 7.6 µL MilQ water and 2 µL cDNA. Then, droplets were generated using droplet generator Bio-rad QX200 (Hercules, California, U.S.A.) and the droplets were transferred to 96 well microplates for PCR in a thermal cycler (Bio-rad C1000 Touch, Hercules, California, U.S.A.). After PCR amplifications, droplets were measured with a droplet reader (Bio-rad QX200, Hercules, California, U.S.A.) to determine absolute gene expression of *fads2* gene. The samples with lesser than 12000 droplets were not used for the gene expression study. The *fads2* gene expression analysis was performed in two replicates for each sample and values were expressed as mRNA copies/µl (Turkmen et al., 2020; Xu et al., 2019).

3.2.5 Liver fatty acid analysis

Liver samples were collected from all the broodfish and stored at -80°C for analysis of fatty acid composition. Crude lipid extraction was carried out with chloroform:methanol (Folch et al., 1957). Hepatic fatty acids methyl esters (FAMES) from total lipids were prepared by transmethylation method with 1% sulfuric acid in methanol (Christie, 1982), purified on NH2

silica (Sep-pak; Waters), and separated and quantified in a gas chromatograph (GC14A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Carbowax 20 M (30 m x 0.32 mm x 0.27 m) silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column initial temperature was set to 170 ºC for 10 min and then it was raised to 220 ºC at 2.5 ºC per min and finally maintained at 215 ºC for a further 5 min. FAMES were identified by comparison with previously characterized standards (Izquierdo et al., 1990). Specific unclear peaks were identified by GLC-MS (TRACE^{TM} GC Ultra and PolarisQ mass spectrometer; Thermo Fisher Scientific).

3.2.6 Ethical statement

The fish broodstock study was conducted as per guidelines of the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was conducted at Aquaculture Research Group (GIA), ECOAQUA, University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain as per the ethical committee norms. All these samplings and study were approved by the Bioethical Committee of the ULPGC *vide* REF: 007/2012 CEBA ULPGC.

3.2.7 Statistical analysis

All results were reported as mean ± standard deviation. All data was checked for normality (Kolmogorov–Smirnoff) and homogeneity (Levene's tests) and if no normality was observed, an arcsine transformation was performed to attain normality. All of the percentage data were arcsine transformed before performing statistical analysis. The regression and Pearson's correlation analysis were performed for studying the relationship between *fads2* expression in PBCs and liver and between sex and broodstock body weight. The independent sample student's *t*-test was performed to compare male (n=4) and female (n=16) broodstock for body weight, Hepato somatic index (HSI%), gonado somatic index (GSI%), PBCs, liver *fads2* expression and hepatic fatty acid composition. Pearson's correlation coefficient was calculated for liver *fads2* expression on hepatic fatty acid of either male or female broodstock. All data was analyzed using the program IBM SPSS version 20 for Windows (IBM SPSS Inc.).

3.3 Results

3.3.1 Biometric and *fads2* expression values for males and females

The GSB male and female broodstock size, HSI%, GSI% and *fads2* expression in PCBs and liver are presented in Table 3-3. As expected, mean body length and weight were significantly higher for females than males (Table 3-3). The mean HSI values for males (1.26±0.17) were 18% higher (P=0.04) than for females (1.08±0.13) (Table 3-3), whereas GSI % values of females (1.47±0.36) were about 2.5 times higher (P<0.001) than for males (0.65±0.16) (Table 3-3). HSI values for each individual fish ranged between 1.06-1.48 and 0.83-1.48% for male and female broodstock, respectively (Figure 3-1), whereas those for GSI ranged from 0.47-0.80 and from 1.01-2.37% for male and female broodstock, respectively (Figure 3-1).

Table 3-3. Gilthead seabream male and female broodstock body length (cm), weight (kg), HSI (%), GSI (%) and PBCs and liver *fads2* expression (mRNA copies/µL) after feeding the low fish meal and fish oil diet for one month

Broodstock	**Males (n=4)**	**Female (n=16)**	***t*-test (*P* value)**
Body length (cm)	47±3[b]	56±4[a]	<0.001
Body weight (kg)	1.27±0.19[b]	2.40±0.63[a]	0.003
HSI (%)	1.26±0.17[a]	1.08±0.13[b]	0.04
GSI (%)	0.65±0.16[b]	1.47±0.36[a]	<0.001
PBCs *fads2* (mRNA copies/µL)	1.68±0.55	2.00±0.93	0.52
Liver *fads2* (mRNA copies/µL)	2.60±0.84	3.24±1.49	0.43

Different superscripts in each row indicate significant differences among male or female broodfish (P<0.05, Independent Sample student's t-test). HSI % (hepato somatic index) = (Liver weight, g/weight of fish, g) x 100; GSI % (gonado somatic index) = (Gonad weight, g/weight of fish, g) x 100.

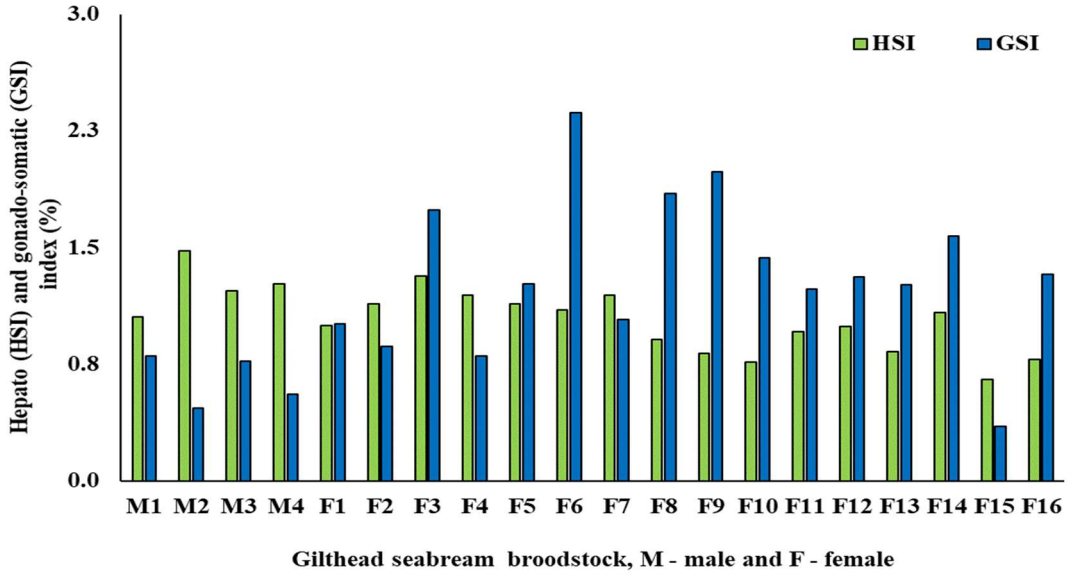

Figure 3-1. Hepato or gonado-somatic index (%) of gilthead seabream male (n=4) or female (n=16) broodstock (M1 to M4 – Male; F1 to F16 – Female).

The mean PBC *fads2* expression of male and females were 1.68±0.55 and 2.00±0.93 copies/µL, respectively and liver *fads2* expression was 2.60±0.84 and 3.24±1.49 copies/µL for male and female broodstock, respectively (Table 3-3). There was a very high coefficient of variation in the *fads2* expression levels in both males (32%) and females (46%). The Student *t*-test showed that the *fads2* expression in PBCs or liver was not significantly (P>0.05) different between males and females, although *fads2* values in PBCs and liver were 20 and 25% higher in females than in males (Table 3-3). No significant correlation was found between body weight and HSI % or between body weight and GSI % of either male or female broodstock (Table 3-4).

Table 3-4. Pearson's correlation of body weight (kg) and HSI or GSI % of male and female gilthead seabream broodstock

Broodstock	Male broodstock		Female broodstock	
	HSI %	GSI %	HSI %	GSI %
Body weight (kg)	-0.35 (0.65)	0.14 (0.86)	0.14 (0.61)	0.25 (0.36)

3.3.2 Comparison of *fads2* expression in PBC or in liver and broodstock body weight

Expression of *fads2* in PBCs was not correlated to body weight in males (r=-0.56; P=0.45), females (r=0.15; P=0.58) nor both sexes (r=0.19; P=0.42) broodstock (Table 3-5). In males,

we found a significant negative correction (r=-0.96; *P*=0.04) between liver *fads2* expression and body weight, whereas in female liver *fads2* expression did not show any correlation (r=0.13; *P*=0.64) to body weight (Table 3-5). Also, we could not find any correlation between broodfish body weight and liver *fads2* expression (r=0.19; *P*=0.43) of both sexes. Moreover, neither *fads2* expression in PBCs nor in liver showed a significant regression with the body weight for males, females or both sex (*P*>0.05; data not showed).

Table 3-5. Pearson's correlation coefficient (PC) of body weight (kg) and PBCs or liver *fads2* expression (mRNA copies/µL) of male, female and both male and female gilthead seabream broodstock

Broodstock	Male broodstock		Female broodstock		Male and female Broodstock	
	PBCs *fads2*	Liver *fads2*	PBCs *fads2*	Liver *fads2*	PBCs *fads2*	Liver *fads2*
Body weight (kg)	-0.56 (0.45)	-0.96 (0.04)	0.15 (0.58)	0.13 (0.64)	0.19 (0.42)	0.19 (0.43)

3.3.3 Relationship between *fads2* expression in PBCs and in liver of male and female broodstock

Individual data on *fads2* expression in PBCs and liver of male and female broodstock are presented in Figure 3-2. The *fads2* expression values in PBCs and liver of male broodstock did not significantly (*P*=0.114) differed (Table 3-6) and Pearson's correlation analysis showed a positive (r=0.76) but not significant (*P*=0.24) correlation between both parameters (Table 3-7). On the contrary, *fads2* expression was significantly lower in PBCs compared to the liver for females (*P*=0.008) and both sexes (*P*=0.003) (Table 3-6). Additionally, female broodstock exhibited a highly positive (r=0.90) and very significant (*P*<0.001) correlation in the *fads2* expression between PBCs and liver. The same trend was observed for PBCs and liver *fads2* gene expression of pooled results for both male and female broodstock (r=0.89; *P*<0.001) (Table 3-7). The regression relationship analysis results for male and female showed that data for females (R^2=0.88; *P*<0.001) and for both sexes combined (R^2=0.85; *P*<0.001) exhibited a significantly higher level of regression between PBCs and liver *fads2* expression than in male broodstock (R^2=0.51; *P*=0.29) (Figure 3-3).

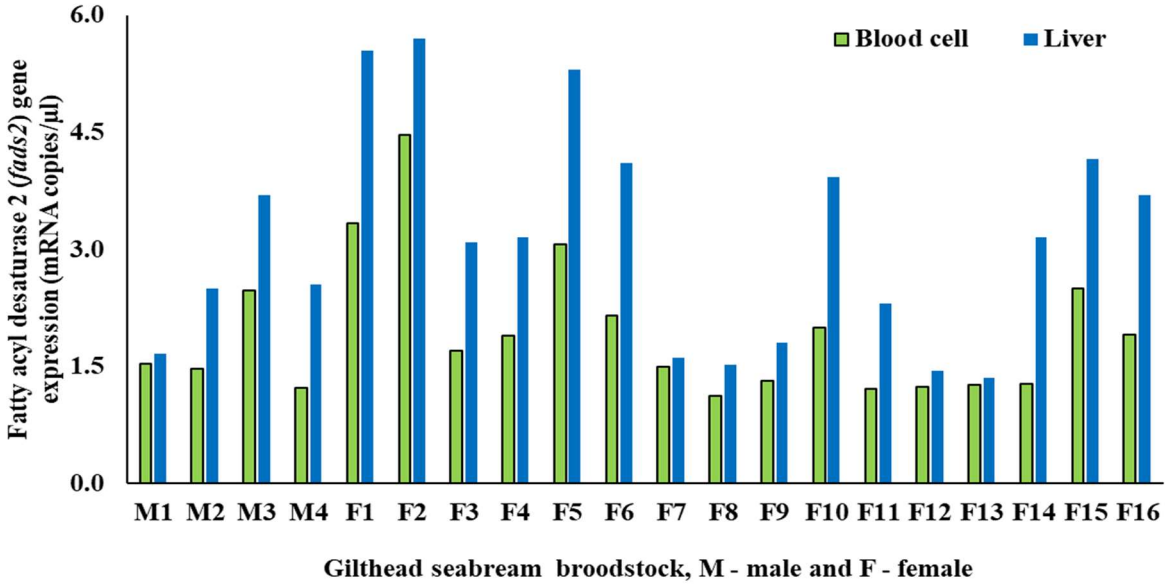

Figure 3-2. Fatty acyl desaturase 2 (*fads2*) mRNA expression (copies/µl) in PBCs or liver of gilthead seabream male (n=4) and female (n=16) broodstock (M1 to M4 – male; F1 to F16 - female)

Table 3-6. Independent sample student's *t*-test of PBCs and liver *fads2* expression (mRNA copies/µL) in male, female and both male and female gilthead seabream broodstock

Broodstock	**PBCs *fads2***	**Liver *fads2***	***t*-test (*P* value)**
Male	1.68±0.55	2.60±0.84	0.114
Female	2.00±0.93[b]	3.24±1.49[a]	0.008
Male and female	1.93±0.87[b]	3.11±1.39[a]	0.003

Different superscripts in each row indicate significant differences ($P<0.05$, Independent sample student's *t*-test).

Table 3-7. Pearson's correlation coefficient (PC) of PBCs and liver *fads2* expression (mRNA copies/µL) in male, female and both male and female gilthead seabream broodstock

	Male broodstock	**Female broodstock**	**Male and female broodstock**
		Liver *fads2*	
PBCs *fads2*	0.76 (0.24)	0.90 (<0.001)	0.89 (<0.001)

3.3.4 Fatty acid composition of the liver

Liver fatty acid profiles were very similar between males and females (Table 3-8). Thus, mean contents in each fatty acid was not significantly different between males and females, except for 16:4n-3 fatty acid, which was 47% higher (P=0.01) in males (Table 3-8). Consequently, the sum of fatty acids belonging to saturated, monounsaturated, n-9, n-6 or n-3 families were not significantly different between males nor females (P>0.05) (Table 3-8). Similarly, there were no significant differences in the ratios among main essential fatty acids (P>0.05) observed (Table 3-8). Fatty acid profiles of individual broodfish markedly differed among individuals, including fatty acids of interest in the bioconversion pathway (Table 3-9). For instance, EPA levels in males ranged between 1.5 to 4.5% and in females between 1.5 to 5.0%, whereas DHA levels were higher than those of EPA with individual values ranging between 5.2 to 13.7% in the males and between 6.0 to 14.4% in the females (Table 3-9). Among males, that one with the highest liver *fads2* expression (M3) and the lowest weight showed the highest hepatic ARA, DHA, total n-3 LC-PUFA or EPA+DHA contents. However, among females, the one with the highest *fads2* expression (F2) did not show the highest levels of these fatty acids (Table 3-9).

To determine the relationship between liver *fads2* expression and hepatic fatty acid composition, a Pearson's correlation analysis was conducted (Table 3-10). In male broodstock, liver *fads2* expression was highly (P<0.05) correlated to 20:3n–6/20:2n–6 (r=0.98) a Fads2 product/precursor ratio and slightly (P<0.1) correlated to 20:3n-9 (r=0.94), 20:3n-6 (r=0.91), 22:4n-6 (r=0.91), 22:5n-6 (r=0.93) and 22:6n-3 (r=0.92), all of them direct or indirect products from Fads2 activity. In females, none of the hepatic FAs showed significant (P>0.05) correlations with liver *fads2* expression.

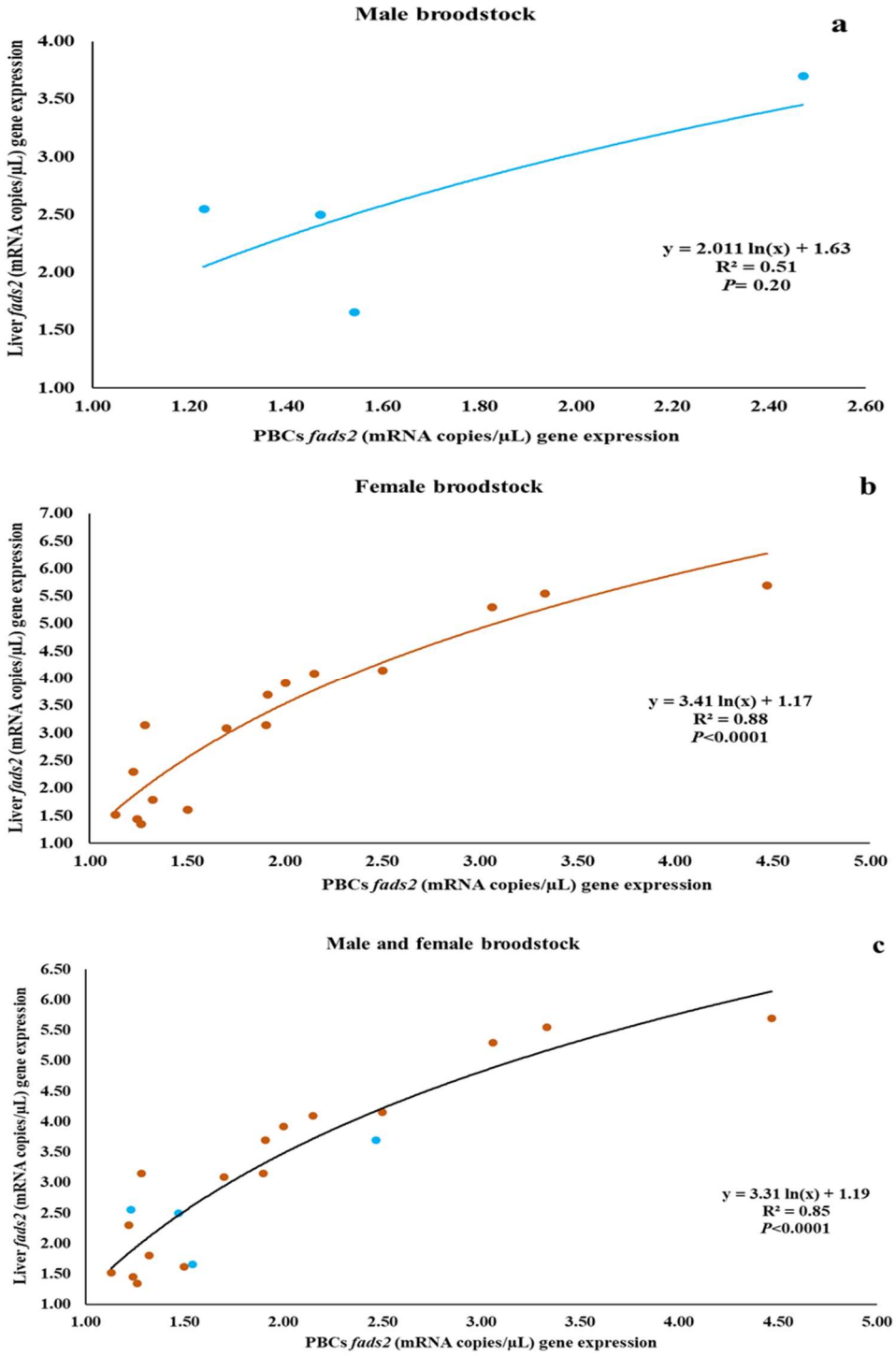

Figure 3-3. The regression relationship between PBCs and liver *fads2* mRNA expression (copies/µl) in gilthead seabream male (n=4) (a), female (n=16) (b) or both (n=20) (c) broodstock.

Table 3-8. Mean hepatic fatty acid composition (% total fatty acids) of male or female gilthead seabream broodstock after feeding the low fish meal and fish oil diet for one month

Fatty acid (% TFA)	Male		Female		*t*-test (*P* value)
	Mean	SD	Mean	SD	
14:0	1.42	0.57	1.30	0.38	0.59
14:1n-5	0.04	0.02	0.03	0.01	0.33
14:1n-7	0.02	0.01	0.03	0.01	0.70
15:0	0.17	0.03	0.14	0.04	0.19
15:1n-5	0.02	0.01	0.02	0.01	0.82
16:0 *iso*	0.02	0.02	0.02	0.01	0.76
16:0	11.56	0.29	11.41	1.42	0.72
16:1n-7	2.48	1.12	2.18	0.46	0.64
16:1n-5	0.06	0.02	0.05	0.01	0.43
16:2n-4	0.14	0.11	0.10	0.04	0.48
17:0	0.14	0.08	0.11	0.04	0.35
16:3n-4	0.16	0.03	0.14	0.02	0.30
16:3n-3	0.06	0.02	0.05	0.01	0.40
16:3n-1	0.04	0.04	0.03	0.01	0.76
16:4n-3	0.17[a]	0.06	0.09[b]	0.04	0.01
18:1n-9	3.86	0.95	3.80	0.45	0.66
18:1n-7	28.81	3.98	29.80	1.61	0.67
18.1n-5	2.69	0.35	2.61	0.15	0.76
18:2n-9	0.11	0.03	0.10	0.03	0.30
18:2n-6 (LA)	0.09	0.05	0.11	0.04	0.71
18:2n-4	14.51	2.52	14.86	1.41	0.77
18:3n-6	0.09	0.04	0.09	0.02	0.61
18:3n-4	0.24	0.07	0.25	0.06	0.56
18:3n-3 (ALA)	0.13	0.03	0.12	0.03	0.43
18.3n-1	9.01	3.24	9.92	1.68	0.45
18:4n-3	0.01	0.01	0.00	0.01	0.72
18:4n-1	0.55	0.29	0.49	0.13	0.82

20:0	0.09	0.04	0.08	0.03	0.45
20:1n-9	0.29	0.02	0.26	0.06	0.71
20:1n-7	0.36	0.18	0.33	0.11	0.66
20.1n-5	2.34	0.72	2.22	0.42	0.86
20:2n-9	0.15	0.05	0.15	0.06	0.24
20:2n-6	0.18	0.05	0.23	0.08	0.34
20:3n-9	0.86	0.18	0.94	0.15	0.88
20:3n-6	0.03	0.01	0.03	0.01	0.60
20:4n-6 (ARA)	0.26	0.16	0.29	0.10	0.74
20:3n-3	0.77	0.64	0.66	0.18	0.17
20:4n-3	0.78	0.28	0.95	0.19	0.76
20:5n-3 (EPA)	0.66	0.17	0.68	0.13	0.75
22:1n-11	2.80	1.26	2.61	1.00	0.50
22:1n-9	1.48	0.92	1.22	0.62	0.97
22:4n-6	0.65	0.18	0.65	0.18	0.35
22:5n-6	0.23	0.13	0.18	0.06	0.56
22:5n-3 (DPA)	0.19	0.08	0.18	0.05	0.66
22:6n-3 (DHA)	2.04	0.78	1.88	0.57	0.61
Total Saturates	9.32	3.73	8.57	2.30	0.66
Total Monoenes	17.43	0.73	17.02	1.77	0.90
Total n-3	39.20	5.71	39.40	1.90	0.94
Total n-6	25.37	3.44	25.24	2.76	0.86
Total n-9	17.05	3.08	17.36	1.39	0.64
Total n-3 LC-PUFA	30.11	4.01	31.15	1.38	0.69
EPA+DHA	15.59	5.04	14.69	3.68	0.62
ARA/EPA	12.12	4.44	11.18	3.11	0.82
EPA/ARA	0.31	0.27	0.28	0.12	0.64
DHA/ARA	5.01	3.05	4.19	1.69	0.66
DHA/EPA	14.72	5.44	13.36	2.97	0.83
DHA/DPA	3.57	1.48	3.46	0.80	0.88

n-3/n-6	4.82	2.20	4.64	0.84	0.74
n-6/n-3	1.52	0.32	1.47	0.26	0.78
18:2n-9/18:1n-9	0.68	0.13	0.70	0.10	0.79
18:3n-6/18:2n-6	0.66	0.54	0.75	0.34	0.86
20:3n-6/20:2n-6	0.02	0.01	0.02	0.01	0.71
18:4n-3/18:3n3	0.29	0.12	0.31	0.09	0.52
20:4n-3/20:3n-3	0.08	0.06	0.05	0.03	0.48

Different superscripts in each row indicate significant differences among male or female broodfish hepatic fatty acids composition ($P<0.05$, Independent sample student's *t*-test).

Table 3-9. Hepatic fatty acid composition (% total fatty acids) of male and female gilthead sea bream broodstock fed a low FM and low FO diet for one month (M1 to M4 – Male; F1 to F16 – Female).

Fatty acid (%TFA)	M1	M2	M3	M4	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
14:0	1.38	2.10	0.71	1.50	0.97	1.75	2.02	1.45	1.01	1.86	1.43	1.37	1.08	0.68	1.25	1.68	0.93	1.18	1.05	1.03
14:1n-5	0.04	0.06	0.02	0.04	0.02	0.04	0.05	0.03	0.03	0.05	0.04	0.04	0.02	0.02	0.02	0.04	0.05	0.02	0.03	0.03
14:1n-7	0.03	0.03	0.01	0.02	0.02	0.03	0.04	0.03	0.02	0.03	0.02	0.02	0.02	0.01	0.01	0.02	0.05	0.04	0.01	0.03
15:0	0.17	0.21	0.13	0.16	0.10	0.17	0.18	0.17	0.10	0.19	0.16	0.17	0.13	0.09	0.10	0.19	0.11	0.12	0.15	0.13
15:1n-5	0.03	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.05	0.04	0.02	0.02
16:0 *iso*	0.00	0.04	0.02	0.03	0.01	0.03	0.03	0.00	0.02	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.04	0.02	0.02	0.02
16:0	11.31	11.54	11.96	11.41	12.59	12.40	12.70	13.72	11.85	11.53	11.96	10.55	12.51	10.12	9.76	12.75	8.25	10.47	10.27	11.20
16:1n-7	2.00	3.77	1.19	2.94	1.93	2.56	2.72	1.80	2.01	3.24	2.38	2.39	1.75	1.44	2.46	2.47	2.35	1.98	1.74	1.73
16:1n-5	0.04	0.07	0.04	0.07	0.04	0.06	0.06	0.03	0.05	0.06	0.05	0.05	0.05	0.04	0.03	0.05	0.06	0.04	0.04	0.04
16:2n-4	0.10	0.28	0.03	0.16	0.07	0.12	0.15	0.06	0.09	0.17	0.12	0.14	0.06	0.05	0.04	0.12	0.15	0.09	0.09	0.08
17:0	0.08	0.25	0.07	0.14	0.07	0.11	0.14	0.07	0.08	0.14	0.10	0.12	0.08	0.06	0.11	0.12	0.16	0.21	0.09	0.08
16:3n-4	0.16	0.18	0.12	0.17	0.13	0.18	0.17	0.12	0.13	0.18	0.16	0.15	0.13	0.12	0.11	0.16	0.15	0.12	0.15	0.14
16:3n-3	0.04	0.08	0.04	0.06	0.04	0.05	0.06	0.04	0.04	0.06	0.05	0.06	0.04	0.04	0.04	0.04	0.08	0.05	0.04	0.05
16:3n-1	0.02	0.03	0.10	0.01	0.02	0.02	0.02	0.03	0.03	0.02	0.03	0.03	0.05	0.04	0.03	0.03	0.06	0.04	0.04	0.04
16:4n-3	0.12	0.26	0.18	0.13	0.07	0.12	0.13	0.05	0.08	0.15	0.10	0.13	0.06	0.05	0.03	0.12	0.17	0.06	0.08	0.08
18:0	3.44	3.08	5.23	3.67	4.42	3.70	4.08	3.87	4.14	3.10	3.48	3.09	4.42	4.28	3.85	3.46	3.09	3.69	3.88	4.17
18:1n-9	31.85	26.36	24.50	32.52	31.34	31.65	30.31	29.40	29.88	29.91	31.28	30.96	28.90	28.06	30.42	31.06	25.57	27.84	30.93	29.36
18:1n-7	2.55	3.08	2.28	2.86	2.62	2.76	2.72	2.36	2.60	2.93	2.66	2.75	2.46	2.45	2.48	2.65	2.71	2.66	2.50	2.44
18:1n-5	0.08	0.15	0.08	0.11	0.08	0.12	0.10	0.08	0.09	0.13	0.10	0.11	0.08	0.08	0.06	0.10	0.18	0.10	0.08	0.11
18:2n-9	0.03	0.07	0.13	0.12	0.10	0.08	0.11	0.05	0.15	0.12	0.06	0.07	0.17	0.12	0.18	0.09	0.12	0.11	0.14	0.10

18:2n-6 (LA)	17.15	11.43	15.85	13.62	14.98	15.18	14.17	17.12	14.21	13.33	15.44	15.53	15.21	14.85	14.83	15.73	11.12	13.76	16.84	15.47
18:2n-4	0.06	0.14	0.06	0.11	0.06	0.09	0.10	0.06	0.08	0.11	0.10	0.10	0.07	0.07	0.06	0.10	0.15	0.09	0.08	0.09
18:3n-6 (GLA)	0.14	0.22	0.31	0.27	0.22	0.18	0.26	0.16	0.29	0.27	0.17	0.20	0.39	0.29	0.23	0.24	0.29	0.26	0.33	0.27
18:3n-4	0.10	0.17	0.10	0.14	0.10	0.12	0.11	0.10	0.12	0.16	0.12	0.14	0.07	0.09	0.08	0.12	0.19	0.13	0.12	0.11
18:3n-3 (ALA)	13.25	5.66	9.54	7.58	10.51	9.90	9.34	13.12	9.22	6.74	10.52	10.31	9.85	10.31	8.86	11.01	6.47	9.48	12.04	11.06
18:4n-3	0.33	0.91	0.29	0.66	0.35	0.49	0.57	0.26	0.51	0.68	0.46	0.55	0.51	0.45	0.38	0.48	0.78	0.42	0.57	0.35
18:4n-1	0.07	0.14	0.04	0.10	0.06	0.09	0.09	0.06	0.06	0.12	0.08	0.10	0.05	0.06	0.05	0.11	0.17	0.08	0.07	0.08
20:0	0.28	0.32	0.29	0.27	0.29	0.21	0.26	0.28	0.24	0.25	0.24	0.27	0.25	0.24	0.22	0.25	0.48	0.27	0.19	0.29
20:1n-9	0.28	0.55	0.15	0.44	0.28	0.33	0.36	0.21	0.31	0.48	0.32	0.39	0.22	0.25	0.28	0.30	0.61	0.45	0.24	0.24
20:1n-7	2.11	3.22	1.50	2.53	2.29	2.08	2.18	1.64	2.13	2.74	2.45	2.51	1.74	2.11	2.42	2.05	3.25	2.41	1.56	1.93
20:1n-5	0.12	0.21	0.10	0.16	0.15	0.14	0.15	0.10	0.13	0.16	0.13	0.14	0.11	0.14	0.16	0.13	0.27	0.32	0.10	0.12
20:2n-9	0.10	0.18	0.22	0.21	0.31	0.16	0.21	0.11	0.37	0.26	0.14	0.15	0.30	0.34	0.25	0.18	0.28	0.22	0.14	0.23
20:2n-6	0.91	0.71	1.08	0.72	1.07	0.84	0.77	0.89	1.01	0.89	1.00	0.88	1.10	1.33	1.02	0.80	0.81	0.77	0.86	1.00
20:3n-9	0.01	0.03	0.04	0.02	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.07	0.05	0.02	0.03
20:3n-6	0.16	0.18	0.50	0.19	0.36	0.18	0.25	0.19	0.48	0.25	0.19	0.15	0.42	0.48	0.34	0.22	0.35	0.28	0.23	0.31
20:4n-6 (ARA)	0.41	0.56	1.72	0.40	0.52	0.44	0.48	0.70	0.75	0.56	0.50	0.47	0.98	1.00	0.76	0.50	0.68	0.65	0.70	0.81
20:3n-3	0.94	0.48	1.08	0.62	1.09	0.84	0.80	0.97	1.01	0.74	0.95	0.85	1.17	1.44	0.83	0.79	0.66	0.89	1.03	1.09
20:4n-3	0.41	0.82	0.71	0.68	0.68	0.57	0.63	0.41	0.82	0.78	0.57	0.65	0.72	0.81	0.81	0.55	0.93	0.73	0.58	0.63
20:5n-3 (EPA)	1.49	4.51	2.41	2.78	1.77	2.23	2.33	1.44	2.46	2.98	2.24	2.57	2.03	2.43	3.31	2.06	5.00	4.82	2.06	2.01
22:1n-11	0.94	2.68	0.61	1.70	0.99	1.10	1.33	0.68	1.15	1.74	1.24	1.52	0.68	0.90	0.85	0.96	3.20	1.58	0.75	0.91
22:1n-9	0.56	0.86	0.46	0.73	0.68	0.55	0.65	0.45	0.71	0.70	0.61	0.63	0.53	0.70	0.53	0.49	1.17	0.93	0.44	0.60
22:4n-6	0.13	0.21	0.42	0.14	0.20	0.14	0.15	0.13	0.18	0.15	0.13	0.15	0.20	0.19	0.16	0.13	0.32	0.24	0.16	0.31
22:5n-6	0.12	0.21	0.29	0.15	0.15	0.14	0.15	0.14	0.21	0.17	0.14	0.13	0.19	0.23	0.18	0.13	0.31	0.19	0.16	0.18

22:5n-3 (DPA)	1.27	3.08	1.69	2.11	1.52	1.75	1.74	1.07	1.90	2.57	1.71	1.96	1.63	2.14	1.86	1.45	3.65	1.88	1.64	1.68
22:6n-3 (DHA)	5.19	10.86	13.69	7.54	6.68	6.25	7.10	6.31	9.16	9.20	6.31	7.40	9.52	11.34	10.51	6.03	14.44	9.82	7.73	9.32
Total Saturates	16.66	17.50	18.39	17.15	18.44	18.34	19.38	19.56	17.42	17.07	17.37	15.57	18.47	15.47	15.29	18.45	13.02	15.94	15.63	16.90
Total Monoenes	40.63	41.06	30.96	44.13	40.46	41.44	40.69	36.83	39.12	42.19	41.30	41.53	36.58	36.21	39.73	40.34	39.52	38.41	38.44	37.56
Total n-3	23.04	26.66	29.63	22.16	22.71	22.20	22.70	23.67	25.20	23.90	22.91	24.48	25.53	29.01	26.63	22.53	32.18	28.15	25.77	26.27
Total n-6	19.02	13.52	20.17	15.49	17.50	17.10	16.23	19.33	17.13	15.62	17.57	17.51	18.49	18.37	17.52	17.75	13.88	16.15	19.28	18.35
Total n-9	32.83	28.05	25.50	34.04	32.73	32.80	31.66	30.23	31.45	31.49	32.43	32.22	30.14	29.49	31.68	32.14	27.82	29.60	31.91	30.56
Total n-3 LC-PUFA	9.30	19.75	19.58	13.73	11.74	11.64	12.60	10.20	15.35	16.27	11.78	13.43	15.07	18.16	17.32	10.88	24.68	18.14	13.04	14.73
EPA+DHA	6.68	15.37	16.10	10.32	8.45	8.48	9.43	7.75	11.62	12.18	8.55	9.97	11.55	13.77	13.82	8.09	19.44	14.64	9.79	11.33
ARA/EPA	0.28	0.12	0.71	0.14	0.29	0.20	0.21	0.49	0.30	0.19	0.22	0.18	0.48	0.41	0.23	0.24	0.14	0.13	0.34	0.40
EPA/ARA	3.63	8.05	1.40	6.95	3.40	5.07	4.85	2.06	3.28	5.32	4.48	5.47	2.07	2.43	4.36	4.12	7.35	7.42	2.94	2.48
DHA/ARA	12.66	19.39	7.96	18.85	12.85	14.20	14.79	9.01	12.21	16.43	12.62	15.74	9.71	11.34	13.83	12.06	21.24	15.11	11.04	11.51
DHA/EPA	3.48	2.41	5.68	2.71	3.77	2.80	3.05	4.38	3.72	3.09	2.82	2.88	4.69	4.67	3.18	2.93	2.89	2.04	3.75	4.64
DHA/DPA	4.09	3.53	8.10	3.57	4.39	3.57	4.08	5.90	4.82	3.58	3.69	3.78	5.84	5.30	5.65	4.16	3.96	5.22	4.71	5.55
n-3/n-6	1.21	1.97	1.47	1.43	1.30	1.30	1.40	1.22	1.47	1.53	1.30	1.40	1.38	1.58	1.52	1.27	2.32	1.74	1.34	1.43
n-6/n-3	0.83	0.51	0.68	0.70	0.77	0.77	0.71	0.82	0.68	0.65	0.77	0.72	0.72	0.63	0.66	0.79	0.43	0.57	0.75	0.70
20:2n-9/20:1n-9	0.36	0.33	1.47	0.48	1.11	0.48	0.58	0.52	1.19	0.54	0.44	0.38	1.36	1.36	0.89	0.60	0.46	0.49	0.58	0.96
18:3n-6/18:2n-6	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.03	0.02	0.02	0.02	0.03	0.02	0.02	0.02
20:3n-6/20:2n-6	0.18	0.25	0.46	0.26	0.34	0.21	0.32	0.21	0.48	0.28	0.19	0.17	0.38	0.36	0.33	0.28	0.43	0.36	0.27	0.31
18:4n-3/18:3n3	0.02	0.16	0.03	0.09	0.03	0.05	0.06	0.02	0.06	0.10	0.04	0.05	0.05	0.04	0.04	0.04	0.12	0.04	0.05	0.03
20:4n-3/20:3n-3	0.44	1.71	0.66	1.10	0.62	0.68	0.79	0.42	0.81	1.05	0.60	0.76	0.62	0.56	0.98	0.70	1.41	0.82	0.56	0.58

Table 3-10. Pearson's correlation of liver *fads2* expression (mRNA copies/µL) and hepatic fatty acid (% TFA) composition of male and female gilthead seabream broodstock.

Fatty acids (%TFA)	Male broodstock		Female broodstock	
	Pearson's correlation	Sig. (*P* value)	Pearson' correlation	Sig. (*P* value)
14:0	-0.57	0.43	-0.07	0.79
14:1n-5	-0.58	0.42	-0.22	0.42
14:1n-7	-0.89	0.11	-0.12	0.66
15:0	-0.57	0.43	-0.20	0.45
15:1n-5	-0.43	0.57	-0.33	0.21
16:0 iso	0.38	0.62	-0.09	0.75
16:0	0.95*	0.04	0.25	0.35
16:1n-7	-0.40	0.60	-0.13	0.62
16:1n-5	-0.11	0.89	-0.04	0.88
16:2n-4	-0.37	0.63	-0.17	0.52
17:0	-0.15	0.85	-0.31	0.24
16:3n-4	-0.70	0.30	0.04	0.87
16:3n-3	-0.11	0.89	-0.33	0.22
16:3n-1	0.85	0.15	-0.48	0.06
16:4n-3	0.32	0.68	-0.19	0.48
18:0	0.83	0.17	0.45	0.08
18:1n-9	-0.75	0.25	0.28	0.29
18:1n-7	-0.42	0.58	0.03	0.90
18.1n-5	-0.10	0.90	-0.19	0.48
18:2n-9	0.86	0.14	0.05	0.85
18:2n-6 (LA)	-0.10	0.90	0.11	0.69
18:2n-4	-0.11	0.89	-0.39	0.14
18:3n-6	0.93	0.07	-0.04	0.89
18:3n-4	-0.11	0.89	-0.14	0.61
18:3n-3 (ALA)	-0.37	0.63	0.10	0.72
18.3n-1	-0.11	0.89	0.20	0.46
18:4n-3	-0.16	0.84	-0.22	0.41

18:4n-1	-0.39	0.61	-0.34	0.20
20:0	0.14	0.86	-0.35	0.18
20:1n-9	-0.40	0.60	-0.24	0.38
20:1n-7	-0.44	0.56	-0.29	0.29
20.1n-5	-0.27	0.73	-0.17	0.52
20:2n-9	0.86	0.14	0.28	0.29
20:2n-6	0.49	0.51	0.19	0.49
20:3n-9	0.94	0.07	-0.14	0.60
20:3n-6	0.91	0.09	0.23	0.40
20:4n-6 (ARA)	0.89	0.11	-0.03	0.91
20:3n-3	0.31	0.69	0.30	0.26
20:4n-3	0.62	0.38	-0.03	0.92
20:5n-3 (EPA)	0.20	0.80	-0.29	0.27
22:1n-11	-0.25	0.75	-0.29	0.27
22:1n-9	-0.33	0.67	-0.12	0.65
22:4n-6	0.91	0.09	-0.06	0.83
22:5n-6	0.93	0.07	-0.11	0.69
22:5n-3 (DPA)	0.12	0.88	-0.21	0.45
22:6n-3 (DHA)	0.92	0.08	-0.21	0.44
Total Saturates	0.97*	0.03	0.28	0.30
Total Monoenes	-0.76	0.24	0.01	0.96
Total n-3	0.80	0.20	-0.26	0.34
Total n-6	0.26	0.74	0.14	0.62
Total n-9	-0.76	0.25	0.31	0.24
Total n-3 LC-PUFA	0.79	0.21	-0.23	0.40
EPA+DHA	0.83	0.17	-0.25	0.36
ARA/EPA	0.72	0.28	0.14	0.61
EPA/ARA	-0.40	0.60	-0.25	0.35
DHA/ARA	-0.45	0.55	-0.21	0.44
DHA/EPA	0.69	0.31	0.20	0.46
DHA/DPA	0.81	0.19	-0.02	0.94

n-3/n-6	0.24	0.76	-0.27	0.31
n-6/n-3	-0.38	0.62	0.21	0.43
18:2n-9/18:1n-9	0.87	0.13	-0.04	0.90
20:2n-9/20:1n-9	0.89	0.11	0.30	0.27
18:3n-6/18:2n-6	0.75	0.25	-0.32	0.23
20:3n-6/20:2n-6	0.98*	0.02	0.15	0.58
18:4n-3/18:3n3	-0.04	0.96	-0.15	0.58
20:4n-3/20:3n-3	0.05	0.95	-0.26	0.32

3.4 Discussion

Fads2 is a rate limiting enzyme involved in the first step of LC-PUFA biosynthesis in all vertebrates. In many marine fish, including gilthead seabream (GSB) its activity is low and the expression of the gene responsible for its production (*fads2*) is very low (Izquierdo et al., 2008; Monroig et al., 2011; Izquierdo et al., 2015; Turkmen et al., 2020). Fads2 has dual Δ6 or Δ8 activities in many marine fish including GSB (Zheng et al., 2004; Turkmen et al., 2017b). An increase in *fads2* expression may result in higher Δ6 Fads production yielding a higher production of essential fatty acids in fish (Turkmen et al., 2017b; Turkmen et al., 2020). The implication of *fads2* gene expression pattern in male and female animals has been studied to elucidate its importance on reproductive performance in higher vertebrates (Childs et al., 2010a; Childs et al., 2010b; Childs et al., 2012; Hoile et al., 2013; Niculescu et al., 2013) and in fish (Turkmen et al., 2020). However, there are no previous studies determining the potential relationship between male and female body weight and *fads2* expression in different tissues. As expected from a protandric hermaphrodite species, females showed a significantly higher body weight than males. However, there were no significant relationships between *fads2* expression in PCBs and body weight of males, females or both sex broodstock were observed. These results are in accordance with our previous studies, in which no correlations were found between either parameters (Turkmen et al., 2020). Moreover, there was no significant correlation of body weight with *fads2* expression in liver of females nor both sexes broodstock were noticed. However, the highest liver *fads2* expression found in male broodstock with the lowest body weight (M3) could be related to individual differences in the genome or epigenome, in agreement with our studies (Turkmen et al., 2020). These results indicated that there is no relationship between broodstock body weight and the relative expression of *fads2* in PBCs or liver.

Our previous studies on reproductive performance of GSB have found that, during the spawning season female broodstock shows higher *fads2* expression in PBC compared to male broodstock (Turkmen et al., 2020). Moreover, in GSB females, there is a significant positive correlation between the plasma 17β-estradiol levels and the *fads2* expression in PBC in accordance with studies reported in mammals (Burdge and Calder, 2005; Childs et al., 2012; Kitson et al., 2013; Chalil et al., 2018). In agreement with the present study, *fads2* expression in PBCs and in liver were respectively 20 and 25% higher in females than in males, but not significantly different. This lack of significance could be due to the fact that the present study was conducted prior to the spawning season, when female gametogenesis was being initiated and GSI still remain low, although significantly higher than that of males. Therefore, 17β-estradiol levels, directly related to the *fads2* expression in PBC, could be expected to be still relatively low. Indeed, fatty acid profiles of liver in females showed 7-28% higher Fads2 derived fatty acids products from n-9 and n-6 series than in male liver. However, n-3 Fads2 derived fatty acids tend to be higher in males. These differences among fatty acid families showed that the fatty acid profile of a tissue is not only related to the desaturation activity. For instance, during exogenous vitellogenesis in female liver, there is an increased synthesis of lipoproteins, particularly phosvitin and lipovitelin rich in n-3 LC-PUFA, which transport lipids to the developing oocyte (Arukwe and Goksøyr, 2003). In agreement with the present study the GSI was significantly (P<0.001) higher in females than in males, whereas the HSI was 18% lower (P=0.04) than in males, suggesting the mobilization of nutrients from liver to gonads. These results agree with the higher GSI observed in female in comparison to males (Zohar et al., 1978; Kissil et al., 2001; Chaoui et al., 2006). In addition, increased *fads2* expression in male liver led to increased contents of 20:3n–6/20:2n–6 a Fads2 product/precursor ratio and slight increase of 20:3n-9, 20:3n-6, 22:4n-6, 22:5n-6 and 22:6n-3, suggesting the increase in lipogenesis pathways. These results agree well with the increased expression of fatty acid synthase gene (*fas*) found in the liver of seabream with increased *fads2* expression (Houston et al., 2017). Higher *fads2* expression in GSB females in comparison to males would be justified by their high EFA requirements during vitellogenesis (Fernández-Palacios et al., 1995; Izquierdo et al., 2015) to supply vital nutrients to the gamete and embryo (Harel et al., 1994; Izquierdo, 1996; Izquierdo et al., 2001; Mazorra et al., 2003; Izquierdo et al., 2015). Besides, LC-PUFA, including ARA and EPA are required as precursors for the production of prostaglandins (Stacey and Goetz, 1982) that regulate steroidogenesis which in turn induce vitellogenin synthesis in liver (Firat et al., 2005; Chaves-Pozo et al., 2008; Migaud et al., 2013). Therefore, the high variability in liver fatty acid profiles of individual brood fish could reflect

the dynamic nature of the fatty acid pool in this metabolically active tissue especially diverting the EFAs towards gametogenesis. Such a specific utilization of EFAs is also in agreement with the suggestions on age or size related increase in requirements for essential fatty acids shown in rodents (Dupont et al., 1972; Hood, 1983; Chapman et al., 2000; Nogalska and Swierczynski, 2001) and fish (Izquierdo et al., 2005).

The high correlation found in broodstock males between liver *fads2* expression and Fads2 products or product/precursor ratio was in agreement with the positive correlation between liver *fads2* mRNA expression and 18:3n-6/18:2n-6 ratio found also in rats (Hofacer et al., 2011). Besides, the positive correlation between 20:3n-6 (dihomo-γ-linolenic acid, DGLA) and ARA with PBCs and / or liver *fads2* expression points out the importance of both fatty acids as precursors of series-1 and series-2 prostaglandins, particularly PGE1 and PGE2, which act as a precursors and are involved in cell signaling, hormone production, and as an anti-inflammatory molecule (Stacey and Goetz, 1982; Abayasekara and Wathes, 1999; Ganga et al., 2006; Cheng et al., 2011). In agreement with our results, peripheral blood FADS2 expression has been shown to have a positive correlation to DGLA fatty acids in humans (Chisaguano et al., 2013). Prostaglandins derived from both fatty acids are found to have a positive effect on testosterone production and sperm quality in fish (Wade and Van Der Kraak, 1993; Asturiano et al., 2000; Asturiano et al., 2001).

In GSB broodstock, *fads2* expression in PBCs has been associated with reproductive performance, suggesting that PBCs *fads2* expression could be a useful biomarker to be considered for broodstock selection (Turkmen et al., 2017b; Turkmen et al., 2020). For instance, GSB expressing higher levels of PBCs *fads2* produces higher amounts of eggs and larvae, as well as juveniles with an improved utilization of low fish meal and fish oil diets (Izquierdo et al., 2015; Turkmen et al., 2017a,b; Turkmen et al., 2019,2020). Additionally, we have observed (unpublished) that male broodstock showing higher PBCs *fads2* expression also have a better sperm quality with increased sperm motility and duration. Peripheral blood mononuclear cells (PBMCs) gene expression has been proposed as a potential biomarker to correlate the expression of the same genes in various tissues that are non-accessible for biopsies to predict health status, physiological condition, stress, disease diagnosis or nutrient metabolism in human and land animals (Liew et al., 2006; Bouwens et al., 2007; Caimari et al., 2010a; Caimari et al., 2010b; de Mello et al., 2012; Halloran et al., 2015). In GSB, PBCs has been also studied as a non-invasive tool to assess mitochondrial energy metabolism

(Martos-Sitcha et al., 2019). However, until now the possible relationship between liver *fads2* and PBCs *fads2* expression has not been addressed. Our study demonstrated that PBCs *fads2* expression is directly related to liver *fads2* expression, since the expression in both types of cells is approximately equal in the males and no correlation was found. On the other hand, a clear correlation between PBCs and liver *fads2* expression was found in female GSB broodstock and highly correlated in the females. Therefore, these results confirm that PBCs *fads2* expression is a relevant non-invasive biomarker to undertake marker-based selection for improved production of EFAs and reproductive performance in fish. Therefore, PBCs *fads2* expression may be a good non-invasive indicator to study the role of *fads2* in growth, body composition, health, or reproduction as well as in studies on fatty acid biosynthesis and metabolism in fish and other animals.

3.5 Conclusions

This study aimed to find the relationship between *fads2* expression pattern in peripheral blood cells and liver of broodstock gilthead seabream, *Sparus aurata* to see the possible use of *fads2* gene as a potential biomarker for the selection of broodstock to undertake breeding programs aiming at improved reproduction, health and nutritional status. We found a highly positive correlation between *fads2* expression levels in the PBCs and liver of GSB broodstock. PBCs *fads2* can be utilized as a valid biomarker for fatty acid metabolism in fish and is applicable to broodstock selection programs. PBCs *fads2* expression levels have a good potential as a non-invasive method to select animals having increased fatty acid bioconversion capability and better ability to deal with fish meal and fish oil free diets.

Author Contributions

Conceived and designed the experiment, M.I., S.F., S.T. and S.K.; Broodstock selection, M.I., S.F., S.T. H.X. and J.M.A.; Diet formulation and preparation, S.F., M.I. and S.K.; Molecular studies, S.F., S.T., M.J.Z. and M.I.; Biochemical studies, S.F. and M.I.; Analysed the data, S.F., M.I. and S.K.; Wrote the paper, S.F., M.I. and S.K. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

This project has received funding from the European Union's Horizon 2020 research, innovation program under grant agreement No 727610 (PerformFISH project). This output reflects the views only of the authors, and the European Union cannot be held responsible for

any use, which may be made of the information contained therein. The first author gratefully acknowledges the financial assistance provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India and study leave by the Director, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India for the doctoral study.

Conflicts of Interest

Authors declare no conflict of interest. The funders had no role in the design of the study, analyses, or interpretation of data or writing of the manuscript, or in the decision to publish the manuscripts.

CHAPTER 4

SCIENTIFIC REPORTS
nature research

Impact factor: 4.379

Article | Open Access | Published: 23 September 2020

Reproductive performance of gilthead seabream (*Sparus aurata*) broodstock showing different expression of fatty acyl desaturase 2 and fed two dietary fatty acid profiles

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Scientific Reports **10**, Article number: 15547 (2020) | Cite this article

932 Accesses | **6** Citations | **1** Altmetric | Metrics

Chapter 4

Abstract

Previous studies have shown that it is possible to nutritionally program gilthead seabream offspring through fish oil (FO) replacement by vegetable oils (VO) in the broodstock diet, to improve their ability to grow fast when fed low fish meal (FM) and FO diets during grow-out phase. However, in those studies broodstock performance was reduced by the VO contained diet. Therefore, the present study aimed to determine if it is possible to replace FO by a mixture of FO and rapeseed oil (RO) with a specific fatty acid profile in broodstock diets, without altering gilthead seabream broodstock reproductive performance. Besides, the study also aimed to evaluate the reproductive performance of broodstock with different expression of fatty acid desaturase 2 gene (*fads2)* a key enzyme in synthesis of long chain polyunsaturated fatty acids. For that purpose, broodfish having either a high (HD) or low (LD) expression of *fads2* were fed for three months during the spawning season with two diets containing different fatty acid profiles and their effects on reproductive hormones, fecundity, sperm and egg quality, egg biochemical composition and *fads2* expression were studied. The results showed that blood *fads2* expression in females, which tended to be higher than in males, was positively related to plasma 17β-estradiol levels. Moreover, broodstock with high blood *fads2* expression showed a better reproductive performance, in terms of fecundity and sperm and egg quality, which was correlated with female *fads2* expression. Our data also showed that it is feasible to reduce ARA, EPA and DHA down to 0.43, 6.6 and 8.4% total fatty acids, respectively, in broodstock diets designed to induce nutritional programming effects in the offspring without adverse effects on spawning quality. Further studies are being conducted to test the offspring with low FM and FO diets along life span.

Keywords: Broodstock, dietary fatty acids, egg quality, *fads2*, nutritional programming

4.1 Introduction

Sustainable development of aquaculture depends much on the efficient use of two limited resources derived from capture fisheries: fishmeal (FM) and fish oil (FO) (FAO, 2018; Tacon and Metian, 2015; Turchini et al., 2019). Great advances have been made to completely replace FM by alternative plant protein sources in diets for both freshwater and marine fish(Hu et al., 2013; Kaushik et al., 2004; Oliva-Teles et al., 2015; Tacon and Metian, 2015; Torrecillas et al., 2017a; Xu et al., 2019). However, total replacement of FO in the diets of marine fish is difficult due to the scarcity of other sources of long chain polyunsaturated fatty acids (LC-PUFA) of the n-3 family, which are essential for purely marine teleost (Izquierdo, 1996; Tocher, 2010). Vegetable oils (VO) have been frequently used to partially replace FO, alone (Izquierdo, 2005; Torstensen et al., 2008) or in combination with FM replacement (Callan et al., 2014; Liang et al., 2014; Simó-Mirabet et al., 2018; Torrecillas et al., 2017b). Replacement of FO by VO is constrained by the lack of n-3 LC-PUFA in VO, despite their high concentrations in 18 carbon (18C) fatty acid precursors, because marine teleost has a limited capacity of bioconversion of 18C fatty acids into LC-PUFA. The first step of n-3 LC-PUFA synthesis in fish is catalysed by delta 6 fatty acid desaturase (Δ6 Fads), which inserts an extra double bond in the precursors, linoleic acid (LA, 18:2n-6) or alpha-linolenic acid (ALA, 18:3n-3). Therefore, this enzyme produces 18:3n-6 and 18:4n-3 and, subsequently, longer carbon chain fatty acids, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) after several elongation, desaturation and β-oxidation steps (Monroig et al., 2011; Tocher, 2015). However, marine fish have a low expression of *fads2*, the gene that codes for Δ6 Fads and, therefore, LC-PUFA must be included in their diet.

In the past years, novel lipid sources high in n-3 LC-PUFA have been studied, such as krill oil (Betancor et al., 2012; Saleh et al., 2015), microalgae (Atalah et al., 2007; Ganuza et al., 2008; Kissinger et al., 2016; Sarker et al., 2016; Sørensen et al., 2016) or the transgenic plant *Camelina sativa* (Betancor et al., 2016; Hixson et al., 2014). However, these n-3 LC-PUFA sources are either still expensive or not produced at sufficient amounts to allow complete replacement of FO in diets for marine fish. One way to optimize the use of these novel lipid sources to completely replace FO would be to combine them with VO high in the 18C precursors and produce marine fish with a higher capacity of LC-PUFA biosynthesis. Two different approaches can be followed to modify LC-PUFA biosynthesis capacity in fish. On the one hand, nutrition during very early life history can markedly affect the ability of organisms to effectively utilize specific nutrients later in life, an effect that is known as

nutritional programming or conditioning (Symonds and Budge, 2009). On the other hand, nutritional manipulation of broodstock diet or selection of broodstock for specific markers of lipid biosynthesis can alter the LC-PUFA biosynthetic capacity (Turkmen et al., 2020). As the gilthead seabream (*Sparus aurata*) (GSB) is a multi-batch spawner, eggs largely depend on the continuous intake of nutrients to complete vitellogenesis during the whole spawning season. Therefore, adequate amounts of essential nutrients, most importantly LC-PUFA must be provided in broodstock diets for the proper gonadal and embryo development (Fernández-Palacios et al., 1995).

Our previous studies have shown that conditioning GSB through specific broodstock diets produces juveniles and adults with a better ability to utilize low FM and FO diets and faster growth (Izquierdo et al., 2015; Turkmen et al., 2017b,2019,2020). In this study, GSB broodstock were fed with four different levels of fatty acid precursors ratio (LA+ALA:n-3 LC-PUFA ratio levels, 7.96:20.52; 32.59:13.13; 41.99:10.60 and 39.97:9.48% total fatty acid) (Izquierdo et al., 2015; Turkmen et al., 2017b). Dietary FO replacement by linseed oil (LO) up to 60% in GSB broodstock diet did not affect the reproductive performance, but further replacement of FO by linseed oil up to 80-100% in broodstock diets for gilthead seabream significantly reduced fecundity, larval quality, and growth of 45 days old fingerlings and 4-month-old juveniles (Izquierdo et al., 2015; Turkmen et al., 2017b). However, offspring from broodstock fed the 100% FO diet showed the best growth and feed utilization even when fed low FM and low FO diets (Izquierdo et al., 2015; Turkmen et al., 2017b). Therefore, it is necessary to find out the optimum levels that allow a nutritional programming effect to improve offspring growth without altering broodstock reproductive performance. Preliminary studies suggest that broodstock with higher *fads2* expression show a higher fecundity than lower *fads2* expressed broodstock (Turkmen et al., 2020), but their specific effect in reproductive success has not been studied in detail. For instance, *FADS2* expression in mammals shows a positive relation with reproductive hormones, such as progesterone or estradiol (Childs et al., 2012). In mice, *fads2* knocked-out lead to an impaired reproductive performance, eventually leading to failure in offspring production (Stoffel et al., 2008). However, there are no specific studies in fish relating the *fads2* expression in broodstock and reproductive hormone levels, reproductive performance or the *fads2* expression in the eggs produced.

The present study aimed to determine if it is possible to replace FO by a mixture of VO and FO that provides LA+ALA: n-3 LC-PUFA ratio levels of 16.1:16.3 in broodstock diets, without altering gilthead seabream broodstock reproductive performance. Secondly, it also

aimed to determine the reproductive performance of broodstock with different *fads2* expression levels and the potential interaction with the broodstock diet. For that purpose, broodfish having either a high (HD) or low (LD) expression of *fads2* were fed for three months during the spawning season with two diets containing different fatty acid profiles. Different parameters of reproductive performance such as plasma steroid hormone levels, fecundity, sperm, and egg quality were recorded, together with the egg biochemical and fatty acid composition and *fads2* expression. The schematic diagram of experimental design is presented in Figure 4-1.

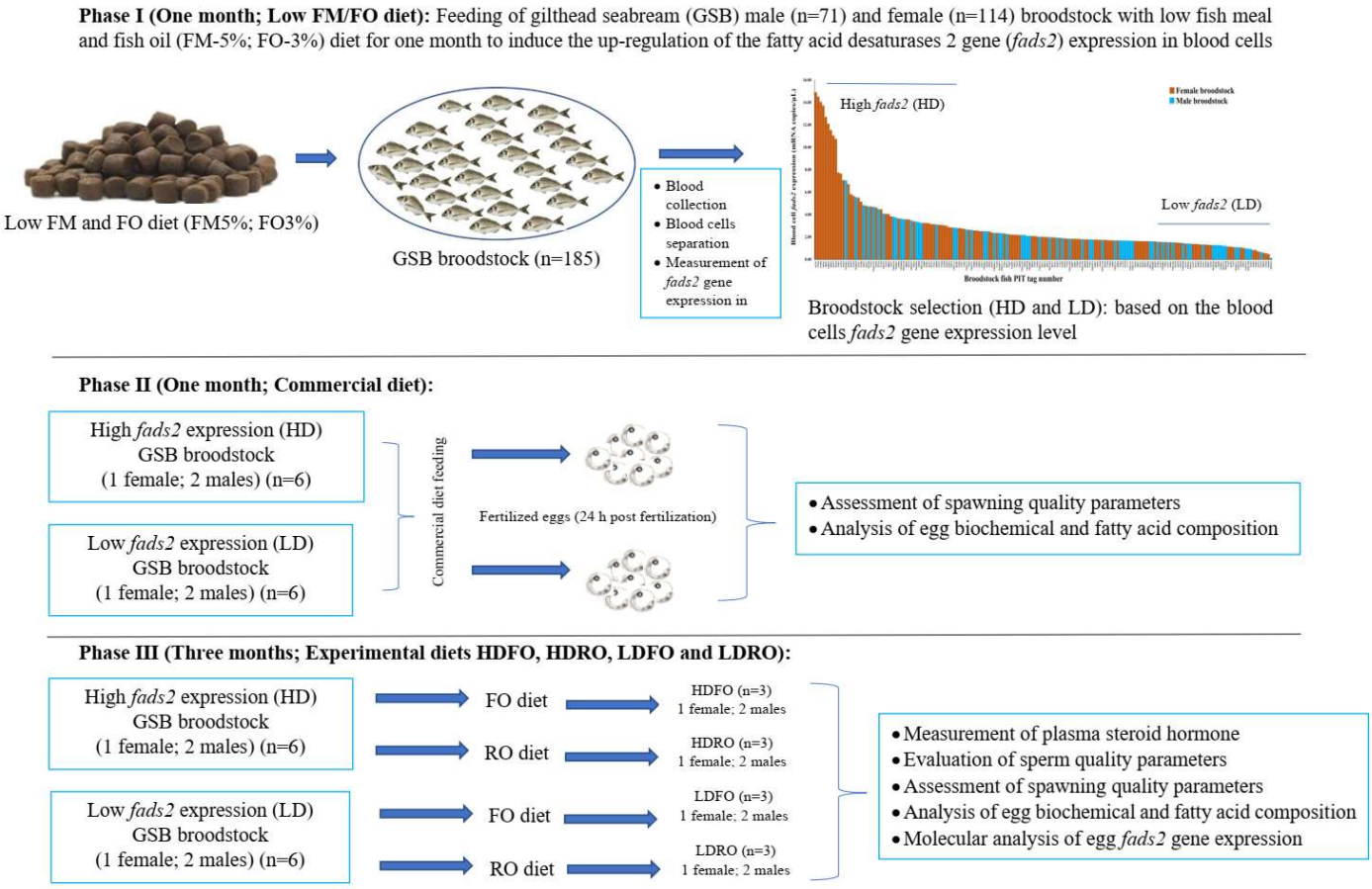

Figure 4-1. Schematic diagram of the study on broodstock selection (*fads2*) and dietary fatty acids profile on reproductive performance in gilthead seabream

4.2 Materials and Methods

4.2.1 Ethical statement

The study was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at GIA, ECOAQUA Institute, University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain. All experimentation performed

at the (ULPGC) was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: 007/2012 CEBA ULPGC).

4.2.2 Characterization of broodstock with high or low *fads2* expression (Phase-I)

The broodstock utilized were obtained as part of a series of long-term ongoing selection programmes involving multiple criteria (Lee-Montero et al., 2013; Navarro et al., 2009). They were reared right from larval stages in our own research facilities of the ECOAQUA Institute. In order to identify broodstock with different ability to synthesize LC-PUFA from LA and ALA, seventy-one 2-year old males (1.02±0.38 kg body weight) and one hundred fourteen 4-year old females (2.07±0.39 kg body weight) were individually tagged with PIT tags (EID Iberica SA-TROVAN, Madrid, Spain) and maintained in a 40 m^3 (5×2.35 m) circular tank. The tanks were supplied with seawater (37 g l^{-1} salinity, 17.8-19.0°C) at a water exchange of 600% daily and maintained under natural photoperiod. Three months before the spawning season, broodfish were fed twice a day for one month with a low FM (5%) and FO (3%) diet (Low FM/FO diet) (Torrecillas et al., 2017b), high in LA and ALA (Table 4-1 and 4-2) and low in LC-PUFA to induce the up-regulation of the fatty acid desaturases 2 gene (*fads2*). After that period, blood samples were collected from all broodfish (n=185) and centrifuged at 3000x*g* for 10 min to separate blood cells and plasma. The blood cell samples were stored at -80°C until RNA extraction. Seabream broodfish were divided into two categories, namely high (HD) or low (LD), based on their *fads2* mRNA copy numbers per µl in blood cells. So, eighteen fish with the highest *fads2* expression values and eighteen with the lowest ones were selected for the conditioning trial (Phase III). Blood cell *fads2* gene expression level was analysed by droplet digital polymerase chain reaction (ddPCR) as previously described (Turkmen et al., 2020; Xu et al., 2019).

Table 4-1. Ingredients and proximate composition of the diet for gilthead seabream broodstock used during Phase-I

Ingredients (%)	Low FM/FO diet
Fish meal[1]	5.00
Blood meal (spray-dried)[2]	7.00
Soya protein concentrate[3]	20.00
Corn gluten meal[4]	22.00
Wheat gluten[4]	5.50
Rapeseed meal[5]	11.30
Wheat[6]	6.89
Fish oil[7]	3.00

Rapeseed oil[5]	5.20
Linseed oil[8]	2.60
Palm oil[4]	5.20
Supplemented ingredients[9]	5.49
Vitamin and mineral premix[10]	0.75
Antioxidant[11]	0.05
Yttrium oxide	0.03
Proximate composition	
Crude protein (% dry matter, DM)	45.1
Crude lipid (%DM)	21.7
Moisture (%)	9.0
Ash (%DM)	5.4

[1]South-American, Superprime – Feed Service Bremen, Germany
[2]Daka, Denmark
[3]Svane Shipping, Denmark
[4]Cargill, Netherlands
[5]Emmelev, Denmark
[6]Hedegaard, Denmark
[7]South American fish oil, LDN Fish Oil, Denmark
[8]Ch. 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)

Table 4-2. Fatty acid profiles of the broodstock diets used in Phases I, II and III (% total fatty acids)

Fatty acid (%TFA)	Phase I	Phase II	Phase III	
	Low FM/FO	Commercial diet	FO diet	RO diet
14:0	6.60	3.50	5.04	1.87
14:1n-5	0.10	0.10	0.15	0.08
15:0	0.10	0.27	0.46	0.17
16:0 ISO	0.00	0.05	0.09	0.09
16:0	12.30	12.46	18.83	9.42
16:1n-7	2.10	3.76	6.84	2.67
16:1n-5	0.10	0.14	0.26	0.11
16:2n-4	0.20	0.34	0.75	0.29
17:0	0.30	0.28	0.83	0.20
16:3n-4	0.10	0.17	0.23	0.17
16:3n-1	0.00	0.12	0.20	0.11
16:3n-3	0.00	0.08	0.12	0.08
16:4n-3	0.40	0.54	1.09	0.43

18:0	3.20	2.42	3.95	2.47
18:1n-9	32.30	32.48	12.82	31.76
18:1n-7	2.30	3.29	3.37	3.28
18:1n-5	0.00	0.15	0.30	0.16
18:2n-9	0.00	0.02	0.19	0.04
18:2n-6 (LA)	20.30	11.51	4.11	11.14
18:2n-4	0.10	0.08	0.24	0.09
18:3n-6	0.10	0.13	0.32	0.13
18:3n-4	0.00	0.07	0.15	0.14
18:3n-3 (ALA)	11.80	5.10	1.30	4.95
18:4n-3	0.40	1.28	2.19	1.22
18:4n-1	0.00	0.06	0.00	0.11
20:0	0.40	0.49	0.47	0.61
20:1n-9	1.00	3.27	3.77	4.06
20:1n-7	0.10	0.17	0.31	0.18
20:2n-9	0.00	0.02	0.06	0.05
20:2n-6	0.10	0.15	0.20	0.17
20:3n-9	0.00	0.04	0.07	0.09
20:3n-6	0.00	0.04	0.12	0.10
20:4n-6 (ARA)	0.20	0.38	1.04	0.43
20:3n-3	0.00	0.08	0.15	0.12
20:4n-3	0.10	0.25	0.57	0.35
20:5n-3 (EPA)	2.50	5.52	11.96	6.57
22:1n-11	0.10	3.45	3.73	4.98
22:1n-9	0.30	0.48	0.51	0.66
22:4n-6	0.00	0.05	0.17	0.23
22:5n-6	0.10	0.11	0.43	0.27
22:5n-3	0.30	0.49	1.40	0.79
22:6n-3 (DHA)	1.70	6.08	11.11	8.42
Σ Saturates	22.90	19.42	29.58	14.74
Σ Monoenes	38.40	47.75	32.12	48.59
Σ n-3	17.20	19.42	29.89	22.93
Σ n-6	20.80	12.38	6.38	12.47
Σ n-3 LC-PUFA	4.60	12.42	25.19	16.25
DHA/EPA	0.68	1.10	0.93	1.28
DHA/ARA	8.50	15.82	10.72	19.82
n-3/n-6	0.83	1.57	4.68	1.84

4.2.3 Comparison of broodstock quality before feeding the conditioning diets (Phase-II)

The selected males and females from HD and LD broodfish were stocked in twelve (6 HD and 6 LD) 1000 L fiberglass tanks with a sex ratio of 2 males to 1 female. Broodstock tanks were supplied with 16L min^{-1} filtered seawater (37±0.5‰ salinity) and strong aeration. At the beginning of the spawning season, from 08 January 2018 to 07 February 2018, fish were fed with a commercial diet (Europa Turbot 18, Skretting, Burgos, Spain) (Tables 4-2 and 4-3)

to ensure that there were no significant differences in the spawning quality among broodfish from the same category (HD and LD). For the evaluation of spawning quality, the spontaneously spawned eggs from each experimental broodstock group were collected four times per week, following this procedure (Fernández-Palacios et al., 1995; Izquierdo et al., 2015; Xu et al., 2019). Eggs were also collected at the end of the feeding period and kept at -80 ºC until biochemical analysis.

Table 4-3. Ingredients and proximate composition of commercial (Phase-II) and experimental diets (FO and RO) used for broodstock nutritional conditioning (Phase-III)

Ingredients (%)	Commercial diet	Exp. Diets	
		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)	57.6	53.4	54.6
Crude lipid (%DM)	17.3	18.8	17.3
Ash (%DM)	9.8	11.3	11.6
Moisture (%)	8.9	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).

4.2.4 Broodstock nutritional conditioning (Phase-III)

From 08 February 2018 to 05 April 2018, the twelve broodstock groups from Phase II were fed one of two different broodstock diets (FO or VO diet), under the same conditions described in the above paragraph. The diets were isoproteic and isolipidic, contained either fish oil (FO) or a mixture of 20% fish oil (FO) and 80% rapeseed oil (RO) and were produced by Skretting ARC (Stavanger, Norway) (Tables 4-2 and 4-3). Compared to the FO diet, the RO diet had higher levels of 18:2n-6 and 18:3n-3 fatty acids and reduced levels of saturated, monoenoic and n-3 LC-PUFA (20:5n-3; eicosapentaenoic acid, EPA and 22:6n-3; docosahexaenoic acid, DHA) (Table 4-2). Fish were fed two times a day (9:00 and 14:00 h) at

1% of their estimated total biomass. Seawater temperature during broodstock spawning period was in the range of 18-22°C (January-April 2018) and fish were kept under natural photoperiod (12 h light). Egg collection for spawning quality and biochemical composition followed the same protocol described in Phase II. Finally, after 30 days of feeding the two different experimental diets, eggs were collected from all broodfish groups (HDFO, HDRO, LDFO, LDRO) and conserved in 1000 µl of RNA Later (Sigma-Aldrich) overnight at 4°C, and then samples were kept at −80 °C until RNA extraction.

4.2.4.1 Plasma steroid hormones

At the end of Phase III, all the GSB broodstock were fasted overnight and anesthetized with clove oil (10 ppm clove oil:methanol (50:50) in sea water) to collect blood samples. Blood was taken from the caudal vein using sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 3.0 mL K3-EDTA tubes (L.P. Italiana, Milan, Italy). Whole blood samples were centrifuged at 3000 g for 10 min at 4°C and plasma was separated and stored at -80°C for sex steroid hormone analyses. Plasma sex steroids were measured by enzyme immunoassays (EIA) as described for European sea bass for testosterone (T) (Rodríguez et al., 2000), 11-ketotestosterone (11-KT) (Rodríguez et al., 2001) and 17β-estradiol (E2) (Molés et al., 2008) and later validated for seabream (Simó-Mirabet et al., 2018). Plasma steroids were extracted with methanol and supernatants were dried and reconstituted in EIA buffer (potassium phosphate 0.1 M, pH 7.4 containing 0.01% sodium azide, 0.4 M NaCl, 0.001 M EDTA and 0.1% BSA). The assays were performed in 96 well plate coated with mouse anti-rabbit IgG monoclonal antibodies (Sigma-Aldrich, R-1008). Steroid standard curves (ranging from 0.0024-5.0 ng/ml for T; 0.0005-1.0 ng/ml for 11-KT and 0.039-80.0 ng/ml for E2; Sigma-Aldrich) or plasma samples were run in duplicate and added to the wells together with the corresponding acetylcholinesterase (AChE) tracer (T-AchE, 11-KT-AChE or E2-AChE; Cayman Chemical, Michigan, USA) and rabbit antiserum (anti-T, anti-11-KT or anti-E2), and incubated at 37 ºC (E2) or 4ºC (T and 11-KT). Next, plates were rinsed, and color development was performed by addition of Ellman reagent. Optical density was read at 405 nm using a microplate reader (Bio-Rad 3550). The sensitivities of the assays (80% of binding) were 0.011 ng/ml for T, 0.0014 ng/ml for 11-KT and 0.31 ng/ml for E2. The inter-assay coefficients of variation at 50% of binding were 10.01% for T, 4.48% for 11-KT and 8.49% for E2. The intra-assay coefficients of variation were 3.78% for T, 3.60% for 11-KT and 1.14% for E2. Sex steroid hormone concentration values are presented as mean ± SD.

4.2.4.2 Sperm quality

At the end of Phase III, all the male broodfish were anesthetized as mentioned above and sperm was collected from the blot dried genital pore after a gentle abdominal massage to induce spermiation and taking care to avoid contamination with water, faeces or urine. The collected sperm was stored on ice until transferred to a 4°C refrigerator. The sperm quality parameters that were evaluated included sperm concentration (number of spermatozoa/ml sperm, 10^9 mL^{-1}), spermatocrit percentage, sperm motility percentage (percentage of spermatozoa showing forward motility) and sperm motility duration (Seconds). Sperm concentration was estimated after a 1000-fold dilution with sperm inactivation media using a Neubauer haematocytometer under 400x magnification. Sperm motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 µl of sperm with 50 µL of seawater (Cabrita et al., 2005; Fauvel et al., 2010; Felip et al., 2009).

4.2.4.3 Egg and larval quality

The collected eggs were placed in 5 L containers provided with aeration, from where 3 randomized 5 ml samples were taken and placed in a Bogorov chamber under the light microscope to calculate the total number of eggs and percentages of fertilized and viable eggs. Egg viability was determined by observing the percentage of morphologically normal eggs after 1-day post fertilization (1 dpf) (Fernández-Palacios et al., 1995; Xu et al., 2019). Then, the viable eggs were individually placed in two replicates in 96-well microtiter plates filled with filtered and sterilized seawater. Eggs were incubated in a controlled temperature incubator at 19-21°C, to estimate the percentage of hatching (2 dpf) and larval survival rates at 3 days post hatch (dph). From these values, the total numbers of fertilized, viable, hatched and larvae produced per kg female were calculated (Fernández-Palacios et al., 1995; Xu et al., 2019).

4.2.5 Biochemical analysis

One month after feeding the commercial diet in Phase-II and one month after the experimental conditioning diets in Phase-III, egg samples were collected from all the broodstock groups and stored at -80°C for analysis of proximate and fatty acid composition. Crude protein content was determined by measuring the N content (N×6.25) through automated Kjeldahl analysis (AOAC, 1995) and crude lipid extraction was carried out with chloroform:methanol (Folch et al., 1957). Fatty acids from total lipids were prepared by transmethylation (Christie, 1982) and separated by gas chromatography (Izquierdo, 1989) and

identified by comparison with previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific). Moisture contents were obtained after drying the samples in an oven at 110°C for 24 h and then for 1 h until constant weight. Ash content was determined after incineration at 600°C for 16 h.

4.2.6 Molecular studies

Total RNA from blood cells (300-400 µl) (Phase I) and egg samples (60-70 mg) (Phase III) was extracted using the RNeasy Mini Kit (Qiagen) and homogenized using the Tissue Lyzer-II (Qiagen, Hilden, Germany) with TRI Reagent (Sigma-Aldrich). Samples were centrifuged with chloroform for phase separation (12,000 g, 15 min, 4°C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into the RNeasy spin column, where total RNA bound to a membrane and RW1 and RPE buffers (Qiagen) were used to wash away contaminants. Purified RNA was eluted with 50 µL of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was undertaken using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The total RNA extraction, cDNA synthesis, primer (*fads2*) designing, and *fads2* gene expression of both blood cells and egg samples were performed as described in our other studies(Izquierdo et al., 2015; Turkmen et al., 2017b,2020; Xu et al., 2019). Primers for *fads2* (Δ6 desaturase) were redesigned in Genetics laboratory of GIA in reference to publications in NCBI (National Center for Biotechnology Information) as follows:

Gene – *fads2* (Δ6 desaturase)
Forward primer sequence: GCA GAG CCA CAG CAG CAG GGA
Reverse sequence: CGG CCT GCG CCT GAG CAG TT

4.2.7 Statistical analysis

Data are reported as mean ± standard deviation. Data were compared statistically using the analysis of variance (ANOVA), at a significance level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene's tests, respectively. Otherwise, an arcsine transformation was performed to attain normality. When arcsine transformed data were not normally distributed, then Kruskal–Wallis non-parametric test was applied to the non-transformed data. An independent sample student's *t*

test was performed to compare egg biochemical and fatty acid composition during Phase-II to check the broodstock selection (HD or LD) effect. One way and two-way ANOVA were applied to the results of sperm and egg and larval quality parameters (total eggs; fertilized eggs; viable eggs; hatched larvae; 3dph larvae per spawn per kg female and fertilization, egg viability, hatching and larval survival rates), egg biochemical and fatty acid composition of phase-III and egg *fads2* expression to determine the combined effects of broodstock selection (HD or LD) and diet (FO or VO). Linear regression analysis was performed for relationships between specific fatty acids contents in eggs (n-3 LC-PUFA) and egg viability or larval survival (3dph) %. All data were analysed using the program IBM SPSS version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Characterization of broodstock with high or low *fads2* expression (Phase-I)

Broodstock body weight was not related to the *fads2* expression levels and HD and LD broodstock had similar body weight (P>0.05) before the experimental period (Table 4-4). The *fads2* expression values in males and females broodstock were in the range of 0.15-7.06 and 0.49-14.92 copies/µL, respectively (Figure 4-2). The mean blood cells *fads2* expression value for males (2.26±3.17copies/µL, n=71) was found to be 32% lower than that of females (3.31±3.16 copies/µL, n=114) for all the selected broodstock (data not shown). Besides, around 10% of the total female broodstock exhibited higher *fads2* (>7.06 copies/µL) expression than any male. In total, 6 females and 12 males with high (HD) or low (LD) *fads2* expression were selected from the highest and lowest *fads2* expression broodstock, their mean values are shown in Figure 4-3. The body weight of the female and male fish which were selected for the next phase (Phase II) showed no significant differences (Table 4-4).

Table 4-4. Body weight (kg) of gilthead seabream male (n=6) and female (n=3) broodstock selected based on blood cells *fads2* expression at the end of Phase I. Different superscripts in a line indicate significant differences among broodfish groups for a given parameter (P<0.05, one-way ANOVA, Tukey Post-Hoc test)

Body weight (kg)	HDFO	HDRO	LDFO	LDRO	*P* value
Male	0.92 ± 0.15	0.85 ± 0.18	0.94 ± 0.16	0.96 ± 0.16	0.73
Female	2.34 ± 0.11	2.29 ± 0.31	2.09 ± 0.23	2.22 ± 0.35	0.83

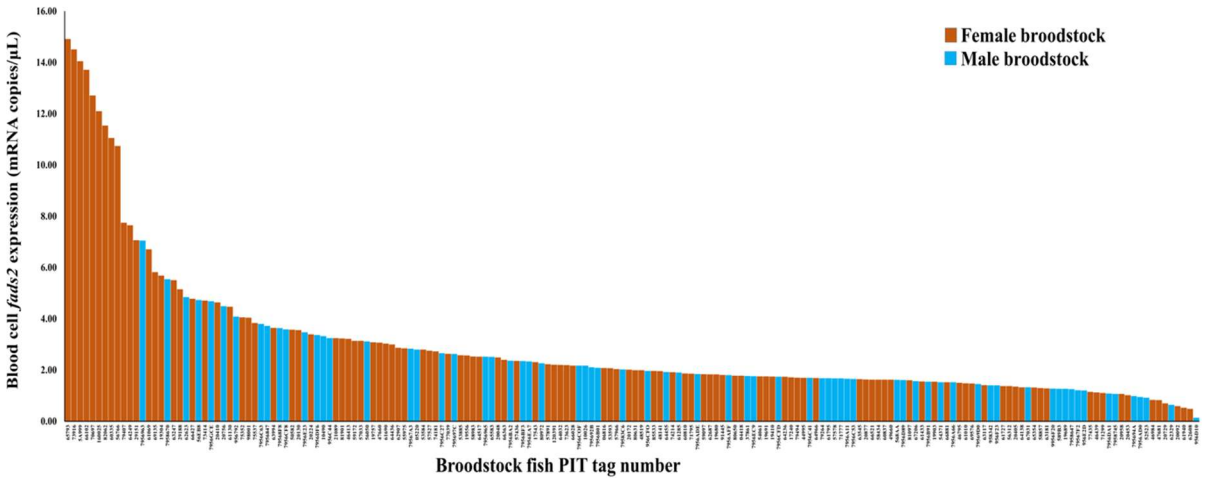

Figure 4-2. Values of *fads2* (mRNA copies/µL) expression in blood cells of gilthead seabream male and female broodstock after being fed the low FM and low FO diet at the end of Phase I.

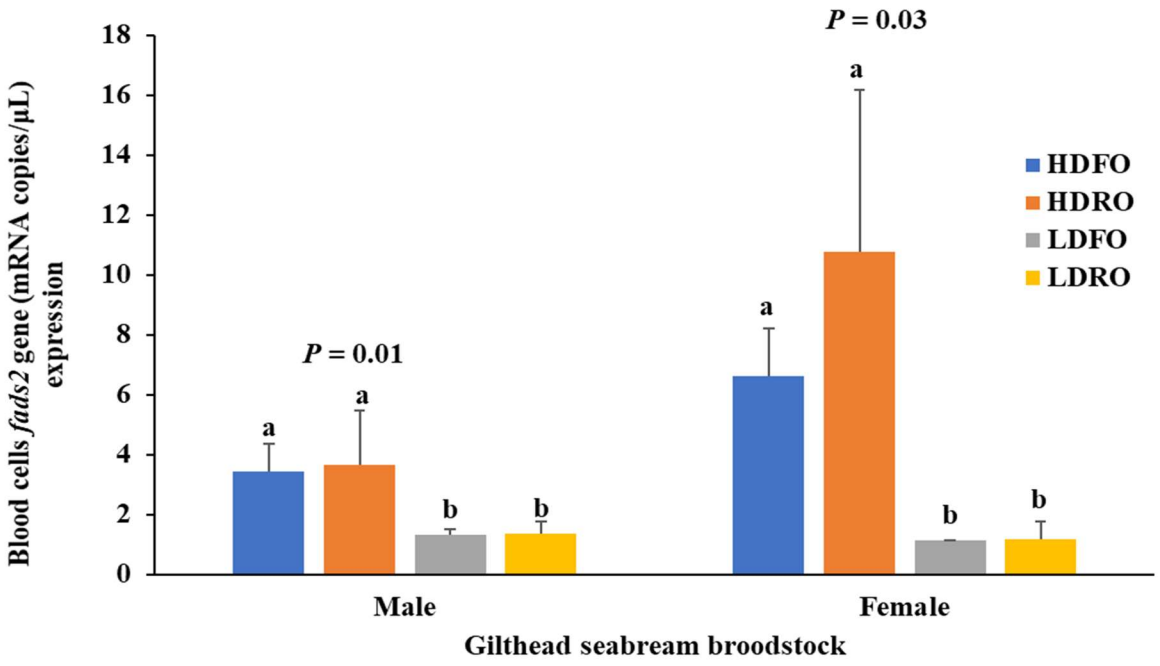

Figure 4-3. Blood cells *fads2* gene (mRNA copies/µL) expression of gilthead seabream male (n=12) and female (n=6) broodstock selected at the end of Phase I and assigned to different groups (HDFO, HDRO, LDFO and LDRO) for subsequent studies. Different superscripts in male or female mean bar indicate significant differences (P<0.05, one-way ANOVA, Tukey Post-Hoc test).

4.3.2 Comparison of broodstock quality before feeding the conditioning diets (Phase – II)

After one month of feeding the commercial broodstock diet (Phase II) at the beginning of the spawning season, no significant differences ($P>0.05$) were found in any of the spawning quality parameters tested among broodfish from the same category (HD and LD) (Supplementary file, Table 4-5). Besides, eggs proximate (Table 4-6) and fatty acid composition (Tables 4-7) also did not show significant ($P>0.05$) differences.

Table 4-5. Quality of egg and larvae obtained from the different gilthead seabream broodstock groups fed with the same commercial diet at the beginning of the spawning season (Phase-II). No significant differences for each spawning quality parameters among broodfish groups (HD *vs* LD) were observed (Independent sample student's *t*-test).

Egg and larval quality parameters	High *fads2* (HD)	Low *fads2* (LD)	*t*-test (*P* value)
Total eggs/spawn/kg female	51,231 ± 17582	51,203 ± 19038	1.00
Fertilization %	81.35 ± 3.70	79.69 ± 19.02	0.92
Egg viability %	76.77 ± 2.94	72.18 ± 20.56	0.81
Hatching %	81.94 ± 12.74	92.72 ± 1.82	0.32
Larval survival (3dph) %	82.57 ± 4.23	84.44 ± 5.91	0.67

Table 4-6. Biochemical composition of eggs obtained from the different gilthead seabream broodstock groups fed with the same commercial diet at the beginning of the spawning season (Phase-II). No significant differences for eggs biochemical composition among broodfish groups (HD *vs* LD) were observed (Independent sample student's *t*-test).

Egg biochemical composition	High *fads2* (HD)	Low *fads2* (LD)	*t*-test (*P* value)
Crude protein (% DM)	67.36 ± 6.03	66.28 ± 2.00	0.77
Crude lipid (% DM)	28.70 ± 2.68	27.95 ± 1.25	0.83
Moisture, %	89.75 ± 0.73	88.87 ± 1.01	0.21

Table 4-7. Fatty acid profiles (% total fatty acids) of gilthead seabream eggs obtained from the different broodstock groups fed with the same commercial diet at the beginning of the spawning season (Phase-II). Different superscripts in each row would indicate significant differences among broodfish groups (HD *vs* LD) (Independent sample student's *t*-test)

Fatty acids (%TFA)	High *fads2* (HD)	Low *fads2* (LD)	*t*-test (*P* value)
14:0	1.09 ± 0.21	1.46 ± 0.50	0.22
14:1n-7	0.19 ± 0.15	0.04 ± 0.01	0.15
14:1n-5	0.22 ± 0.17	0.08 ± 0.02	0.21
15:0	0.27 ± 0.11	0.21 ± 0.03	0.46
15:1n-5	0.20 ± 0.13	0.05 ± 0.02	0.11
16:0 ISO	0.23 ± 0.12	0.09 ± 0.03	0.13
16:0	10.45 ± 0.95	12.56 ± 1.60	0.08
16:1n-7	2.79 ± 0.34	3.34 ± 0.60	0.18
16:1n-5	0.25 ± 0.21	0.10 ± 0.02	0.31
16:2n-4	0.27 ± 0.11	0.20 ± 0.03	0.31

17:0	0.26 ± 0.12	0.16 ± 0.02	0.23
16:3n-4	0.31 ± 0.18	0.21 ± 0.00	0.38
16:3n-3	0.28 ± 0.24	0.11 ± 0.02	0.29
16:3n-1	0.30 ± 0.31	0.10 ± 0.03	0.33
16:4n-3	0.36 ± 0.37	0.16 ± 0.03	0.41
18:0	3.17 ± 0.43	3.39 ± 0.28	0.48
18:1n-9	22.41 ± 2.26	25.23 ± 0.82	0.10
18:1n-7	2.66 ± 0.21	2.90 ± 0.06	0.12
18.1n-5	0.27 ± 0.19	0.15 ± 0.02	0.36
18:2n-9	0.26 ± 0.17	0.18 ± 0.06	0.51
18.2n-6 (LA)	11.08 ± 1.02	12.34 ± 0.79	0.14
18:2n-4	0.26 ± 0.11	0.15 ± 0.01	0.14
18:3n-6	0.56 ± 0.36	0.31 ± 0.09	0.27
18:3n-4	0.50 ± 0.25	0.22 ± 0.06	0.12
18:3n-3 (ALA)	2.38 ± 0.20	2.66 ± 0.15	0.10
18:4n-3	0.60 ± 0.10	0.64 ± 0.02	0.49
18:4n-1	0.31 ± 0.15	0.15 ± 0.03	0.12
20:0	0.25 ± 0.12	0.13 ± 0.04	0.16
20:1n-9	0.29 ± 0.09	0.18 ± 0.03	0.11
20:1n-7	1.33 ± 0.12	1.18 ± 0.14	0.20
20:1n-5	0.34 ± 0.09	0.19 ± 0.02	0.06
20:2n-9	0.32 ± 0.21	0.12 ± 0.02	0.16
20:2n-6	0.69 ± 0.15	0.54 ± 0.06	0.15
20:3n-9	0.28 ± 0.24	0.06 ± 0.01	0.17
20:3n-6	0.54 ± 0.32	0.22 ± 0.07	0.17
20:4n-6 (ARA)	0.82 ± 0.05	0.79 ± 0.06	0.57
20:3n-3	0.56 ± 0.28	0.36 ± 0.04	0.27
20:4n-3	0.89 ± 0.06	0.78 ± 0.02	0.06
20:5n-3 (EPA)	5.43 ± 0.66	5.76 ± 0.45	0.49
22:1n-11	0.55 ± 0.22	0.36 ± 0.05	0.19
22:1n-9	0.44 ± 0.24	0.22 ± 0.02	0.16
22:4n-6	0.55 ± 0.43	0.12 ± 0.02	0.14
22:5n-6	0.68 ± 0.54	0.26 ± 0.02	0.22
22:5n-3	3.37 ± 0.35	2.91 ± 0.42	0.17
22:6n-3 (DHA)	20.77 ± 1.28	18.54 ± 2.68	0.20
Σ Saturates	15.48 ± 1.01	17.93 ± 1.85	0.07
Σ Monoenes	31.91 ± 1.34	34.02 ± 1.09	0.08
Σ n-3	34.62 ± 1.39	31.92 ± 3.42	0.20
Σ n-6	14.89 ± 0.93	14.59 ± 0.60	0.64
Σ n-3 LC-PUFA	31.01 ± 1.77	28.35 ± 3.59	0.25
DHA/EPA	3.86 ± 0.37	3.21 ± 0.22	0.04
DHA/ARA	25.47 ± 0.57	23.41 ± 1.94	0.20
n-3/n-6	2.33 ± 0.22	2.20 ± 0.33	0.53

4.3.3 Broodstock nutritional conditioning (Phase-III)

4.3.3.1 Plasma steroid hormones

Analysis of plasma steroid hormones after feeding the experimental diets denoted no significant differences in plasma testosterone, 11-ketotestosterone or 17β-estradiol levels among the different broodstock groups (Table 4-8). The highest 11-ketotestosterone levels were found in broodstock with the smallest weight and a significant negative linear regression relation was found between broodstock body weight and 11-ketotestosterone levels (R^2=0.517; P<0.001) (Figure 4-4a). The opposite relation was found with the 17β-estradiol levels that showed a significant positive exponential relation with the broodstock body weight (R^2=0.464; P<0.001) (Figure 4-4b). No relation was found between plasma steroid hormones and broodstock blood cells *fads2* expression when all broodstock data were compared. However, in females with low *fads2* expression, there was a positive linear regression relation but no significant difference between plasma 17β-estradiol levels and blood cells *fads2* expression (R^2=0.502; P=0.115) was observed.

Table 4-8. Plasma steroid hormone levels of male (n=12) and female (n=6) gilthead seabream broodstock with high (HD) or low (LD) *fads2* expression fed with either FO or RO experimental diets during Phase-III. Different superscripts in a line would indicate significant differences among broodfish groups for a given parameter (P<0.05, one-way ANOVA, Tukey Post-Hoc)

Plasma steroid hormones (ng/ml)	Sex	HDFO	HDRO	LDFO	LDRO	*P* value
Testosterone	Male	0.341±0.064	0.482±0.221	0.562±0.216	0.418±0.197	0.55
	Female	1.074±1.138	0.332±0.414	0.252±0.093	0.285±0.011	0.54
11 Keto-testosterone	Male	0.059±0.049	0.073±0.026	0.071±0.029	0.089±0.022	0.55
	Female	0.039±0.049	0.011±0.006	0.008±0.001	0.006±0.000	0.57
17β-estradiol	Male	0.434±0.467	0.196±0.081	0.332±0.187	0.185±0.055	0.29
	Female	3.044±1.107	1.019±0.985	1.444±0.943	1.590±1.080	0.35

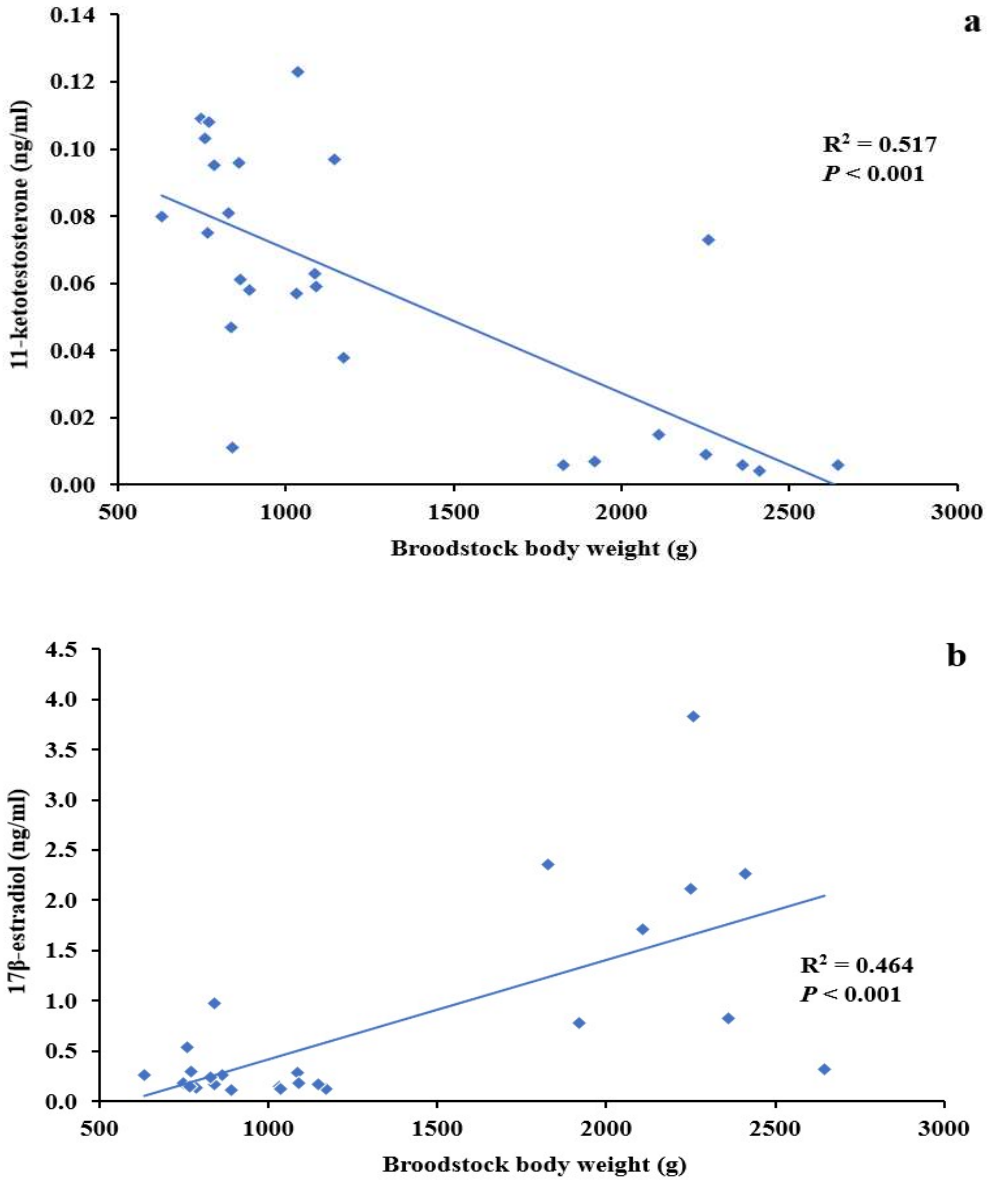

Figure 4-4. The linear regression relationship between body weight and plasma levels of 11-ketotestosterone (a) or 17β-estradiol (b) in gilthead seabream broodstock at the end of Phase III.

4.3.3.2 Sperm quality

After one month of feeding the experimental conditioning diets, sperm concentration or motility duration were not influenced by the *fads2* expression in the broodstock neither by the dietary fatty acids profile or their combination as denoted by the two-way ANOVA analysis (P>0.05) (Table 4-9). However, the percentage of sperm showing motility after activation with seawater was around 10% higher in broodstock with higher *fads2* expression, indicating the

effect of *fads2* expression in broodstock on sperm motility (Table 4-9). Indeed, a highly significant positive linear regression relation was found between *fads2* expression levels in broodstock males and sperm motility (R^2=0.70; *P*=0.003) (Figure 4-5). Besides, a positive linear regression relation but no significant difference was observed between the male testosterone levels and the sperm concentration (R^2=0.823; *P*=0.093). No other relation was found between sperm quality and male hormones plasma levels.

4.3.3.3 Egg and larval quality

Before feeding the experimental conditioning diets neither broodstock quality nor egg biochemical composition differed among experimental broodstock (Table 4-5, 3-6 and 4-7). However, after feeding the experimental diets, fish fecundity, measured as mean number of eggs/spawn/kg female, was the highest in HDFO broodstock and the lowest in LDRO broodstock (Figure 4-6; Table 4-10). Thus, the two-way ANOVA analysis showed a significant (*P*<0.05) improvement in fecundity in broodstock with higher *fads2* expression, whereas feeding RO did not significantly (*P*>0.05) affected broodstock fecundity. Other spawning quality parameters such as fertilization, hatching or larval survival rates were not affected by neither broodstock *fads2* expression nor by the broodstock conditioning diet or their combination (Table 4-10). Only egg viability rate was slightly improved in broodstock with higher *fads2* expression (*P*=0.07) (Table 4-10). Besides, a significant linear relation (R^2=0.397; *P*=0.05) was found between sperm motility and egg viability percentage (Figure 4-7). A significant linear regression relationship was observed between female broodstock blood cells *fads2* expression and all the spawning quality parameters (Figure 4-8). No interaction between diet or broodstock *fads2* expression was detected by the two-way ANOVA analysis (Table 4-10). As a consequence, the number of fertilized eggs/spawn/kg female (*P*<0.05) and particularly, the numbers of viable eggs/spawn/kg female, hatched larvae/spawn/kg female or larval survival 3 dph/spawn/kg female were the highest in HDFO broodstock and the lowest in LDRO (Figure. 4-6).

Table 4-9. Sperm quality (n=6) from the different gilthead seabream broodstock groups fed either the FO or the RO experimental diets (Phase-III). No significant differences for sperm quality parameters among broodfish groups were observed ($P<0.05$, one-way ANOVA, Tukey Post-Hoc).

Sperm quality parameters	Broodfish groups				Two-way ANOVA *P* values		
	HDFO	HDRO	LDFO	LDRO	Broodstock *fads2* expression	Diet	Broodstock *fads2* expression x Diet
Sperm concentration (10^9 sperm/ml)	8.63 ± 1.22	9.96 ± 0.60	11.12 ± 0.23	9.28 ± 2.34	0.38	0.80	0.15
Spermatocrit percentage	77.50 ± 3.54	70.00 ± 13.23	55.00 ± 0.00	71.67 ± 7.64	0.12	0.46	0.08
Sperm motility percentage	92.50 ± 3.54	93.33 ± 2.89	82.50 ± 3.54	83.33 ± 5.77	0.01	0.77	1.00
Sperm motility duration (Second)	810 ± 127	780 ± 312	630 ± 127	760 ± 277	0.56	0.77	0.64

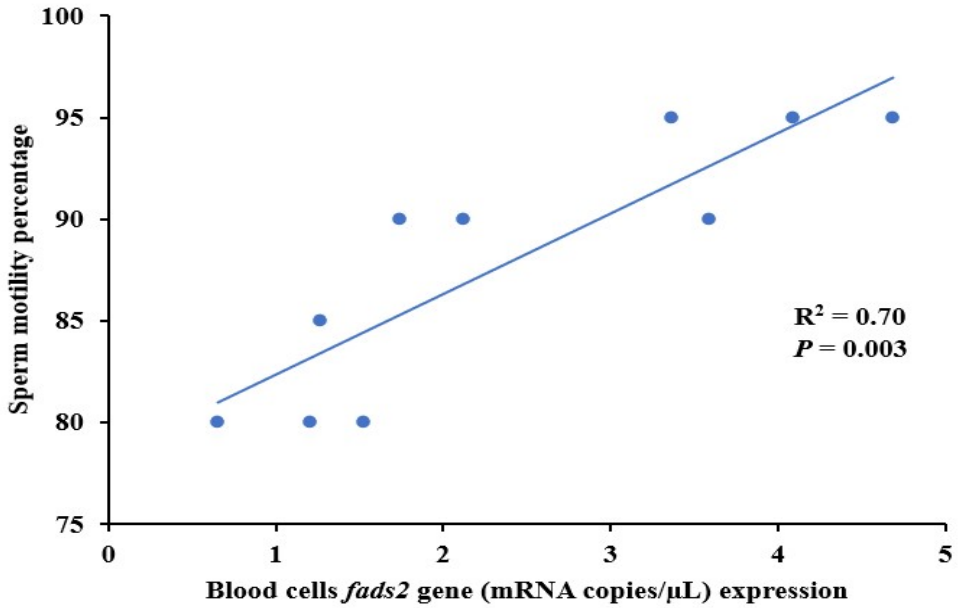

Figure 4-5. The relationship between blood cells *fads2* expression (end of Phase I) and sperm motility percentage in male (n=10) gilthead seabream of the high or low *fads* groups and fed either the fish oil (FO) or the rapeseed oil (RO) diets measured at the end of Phase III.

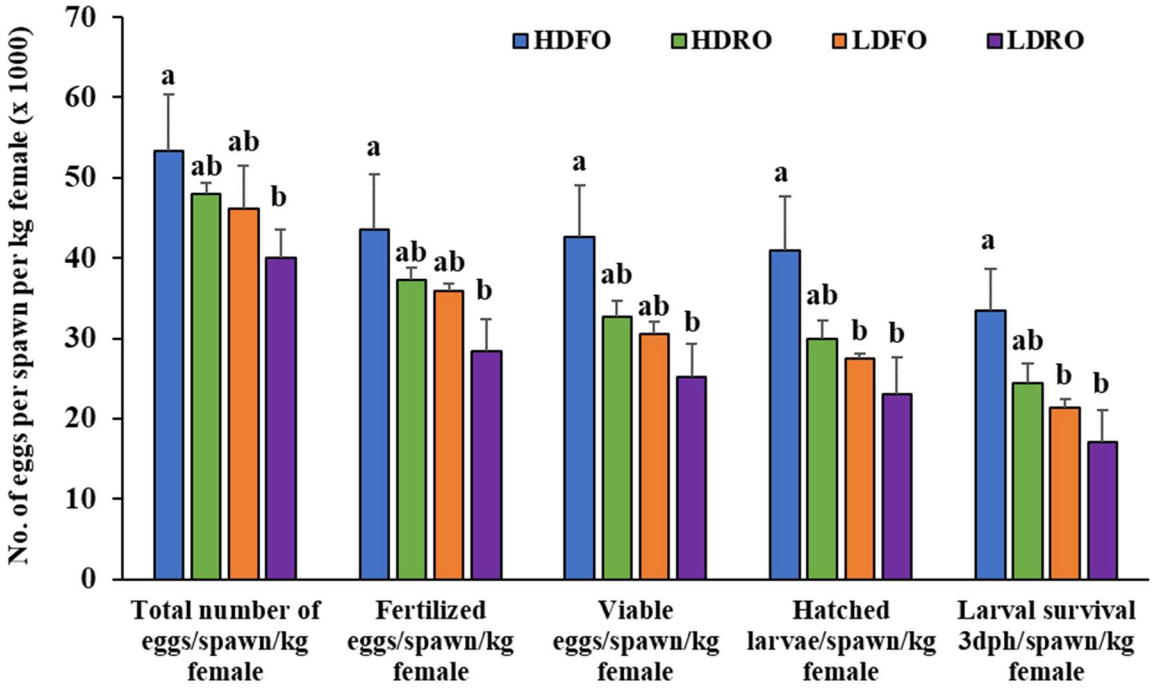

Figure 4-6. Reproductive performance of gilthead seabream broodstock (n=76 spawns over three months) with high (HD) or low (LD) *fads2* expression fed with either FO or RO diet over three months (Phase-III). Different superscript letters for each spawning quality parameters differ significantly (P<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 4-10. Quality of egg and larvae (n=76 spawns per three months) obtained from the different gilthead seabream broodstock groups fed either the FO or the RO experimental diets (Phase-III). Different superscripts in a line indicate significant differences among broodfish groups for a given parameter (P<0.05, one-way ANOVA, Tukey Post-Hoc)

Egg and larval quality parameters	Broodfish groups				Two-way ANOVA *P* values		
	HDFO	HDRO	LDFO	LDRO	Broodstock *fads2* expression	Diet	Broodstock *fads2* expression x Diet
Nº of eggs/spawn/kg female	53,398 ± 6,943^{a}	48,040 ± 1323^{ab}	46,086 ± 5458^{ab}	40,044 ± 3464^{b}	0.03	0.08	0.90
Fertilization %	80.34 ± 2.57	76.19 ± 6.21	77.50 ± 12.51	70.51 ± 6.73	0.47	0.29	0.72
Egg viability %	78.61 ± 2.55	66.35 ± 6.73	65.39 ± 5.06	62.22 ± 7.60	0.07	0.09	0.26
Hatching %	96.04 ± 1.47	90.23 ± 4.11	89.50 ± 2.41	90.66 ± 3.76	0.15	0.27	0.13
Larval survival (3dph) %	81.72 ± 0.59	76.27 ± 5.43	75.69 ± 2.23	71.96 ± 6.66	0.14	0.21	0.71

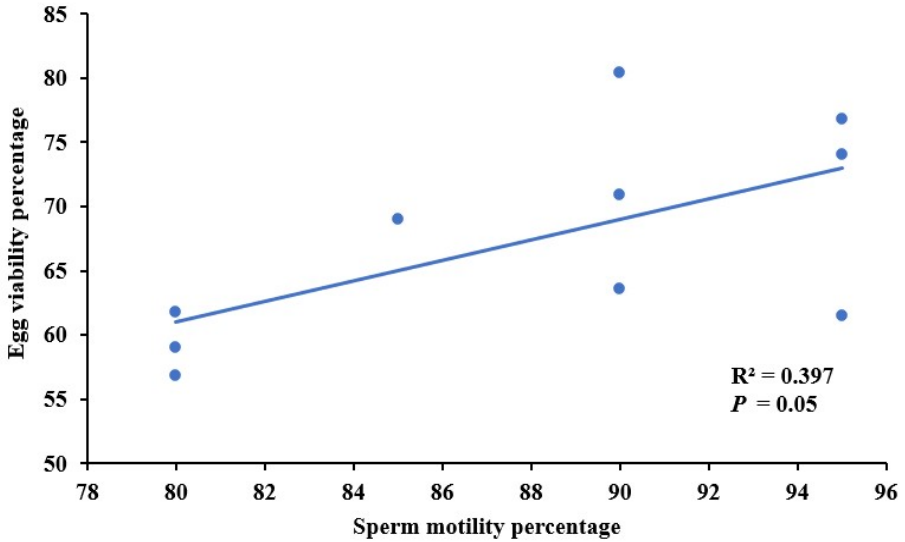

Figure 4-7. The relationship between sperm motility and egg viability percentage (n=10) of the different groups (HDFO, HDRO, LDFO and LDRO) of gilthead seabream broodstock, assessed at the end of Phase-III.

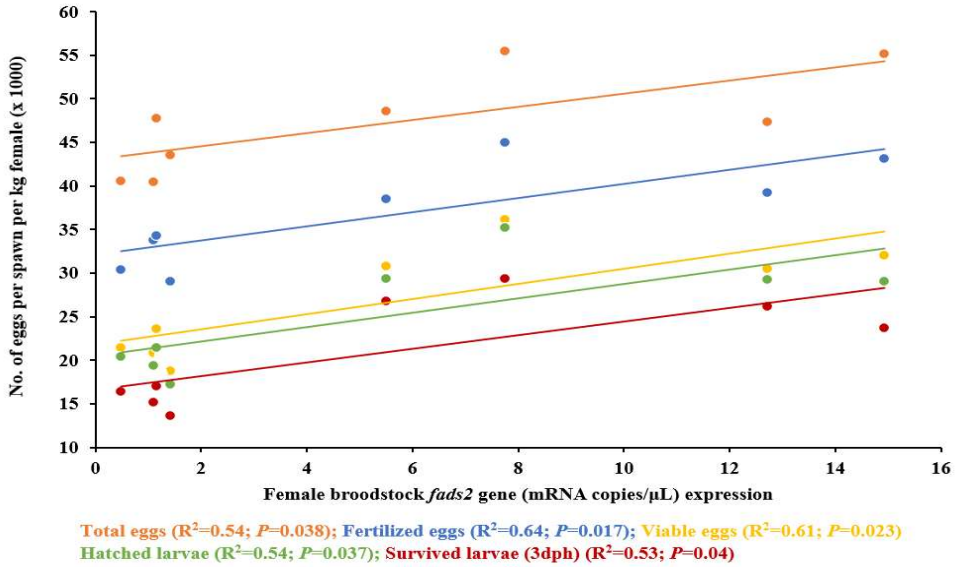

Figure 4-8. The linear regression relationship between female blood cells *fads2* expression (measured at the end of the preparatory Phase I) and spawning quality (n=8) of the different groups (HDFO, HDRO, LDFO and LDRO) pf gilthead seabream broodstock over the whole Phase III.

4.3.3.4 Egg biochemical composition

Proximate composition of eggs was not significantly (P>0.05) affected either by the *fads2* expression in broodstock or the experimental conditioning diet (Table 4-11). However, the two-way ANOVA analysis showed that eggs from broodstock with a higher *fads2* expression (HDFO and HDRO) showed a significant (P<0.05) increase in 20:3n-9, 22:5n-3, DHA/ARA, n-3/n-6, and, particularly, 22:6n-3 (40% average increase), and a mild (P<0.10) increase in 16:3n-3, 16:4n-3, 18:4n-3 and DHA/EPA (Table 4-12). Besides, the relative contents in 14:0, 14:1n-7, 14:1n-5, 15:0, 15:1n-5, 16:0 ISO, 16:0, 16:1n-7, 16:1n-5, 16:2n-4, 17:0, 18:1n-9, 18:2n-4 and 22:4n-6 were reduced in eggs from broodstock with a higher *fads2* expression (Table 4-12). As expected from their dietary levels, the two-way ANOVA analysis showed that feeding broodstock with RO diet (HDRO and LDRO) significantly (P<0.05) increased 18:1n-9, 18:2n-6, and 18:3n-3 (60% average increase) as well as total monounsaturated and n-6 fatty acids (Table 4-12). Besides, feeding RO diet reduced (P<0.05) the levels of 16:1n-7, 17:0, 20:4n-6, 20:5n-3, 22:6n-3 (30% average reduction) as well as those of total saturated, n-3 and n-3/n-6 fatty acids (Table 4-12). Both egg viability (Figure. 4-9a) and larval survival (Fig. 4-9b) at 3 dah showed highly significant positive linear regression relationship to the total n-3 LC-PUFA content in the egg (y =0.787x + 43.423, R^2 = 0.98, P=0.012; and y = 0.4384x + 62.639, R^2=0.97, P=0.015, respectively).

Table 4-11. Biochemical composition (n=3) of gilthead seabream eggs obtained from the different broodstock groups fed either the FO or the RO experimental diets (Phase-III). No significant differences for eggs biochemical composition among broodfish groups were observed ($P<0.05$, one-way ANOVA, Tukey Post-Hoc)

Egg biochemical composition	Broodfish groups				Two-way ANOVA *P* values		
	HDFO	HDRO	LDFO	LDRO	Broodstock *fads2* expression	Diet	Broodstock *fads2* expression x Diet
Crude protein (% DM)	77.23 ± 0.51	72.74 ± 1.77	74.18 ± 4.49	73.73 ± 2.17	0.46	0.17	0.22
Crude lipid (% DM)	21.48 ± 2.11	21.71 ± 1.21	22.45 ± 2.60	23.88 ± 3.26	0.34	0.67	0.89
Moisture (%)	89.09 ± 1.29	89.00 ± 3.26	91.77 ± 0.10	90.78 ± 0.64	0.17	0.75	0.67

Table 4-12. Fatty acid composition (expressed as % total fatty acids) of gilthead seabream eggs (n=3) from the different broodstock groups fed either the FO or the RO experimental diets (Phase-III). Superscripts in a line indicate significant differences in concentrations for a given fatty acid ($P<0.05$, one-way ANOVA, Tukey Post-Hoc)

Fatty acids (%TFA)	Broodfish groups				Two-way ANOVA *P* values		
	HDFO	HDRO	LDFO	LDRO	Broodstock *fads2* expression	Diet	Broodstock *fads2* expression x Diet
14:0	1.48 ± 0.11	1.18 ± 0.12	3.85 ± 0.17	3.43 ± 1.71	0.01	0.60	0.93
14:1n-7	0.04 ± 0.01^{ab}	0.02 ± 0.02^{b}	0.11 ± 0.02^{ab}	0.16 ± 0.06^{a}	0.01	0.47	0.20
14:1n-5	0.07 ± 0.01	0.05 ± 0.01	0.17 ± 0.01	0.23 ± 0.11	0.02	0.64	0.43
15:0	0.21 ± 0.01^{b}	0.17 ± 0.01^{b}	0.43 ± 0.01^{a}	0.38 ± 0.08^{a}	0.00	0.18	0.82
15:1n-5	0.04 ± 0.01^{b}	0.03 ± 0.01^{b}	0.13 ± 0.01^{ab}	0.21 ± 0.06^{a}	0.00	0.19	0.08
16:0 ISO	0.10 ± 0.01^{b}	0.04 ± 0.00^{b}	0.09 ± 0.01^{b}	0.20 ± 0.05^{a}	0.01	0.17	0.00
16:0	12.11 ± 0.49^{b}	12.10 ± 0.92^{b}	19.74 ± 0.78^{a}	15.14 ± 2.29^{ab}	0.00	0.05	0.05
16:1n-7	3.71 ± 0.57^{bc}	2.73 ± 0.07^{c}	5.69 ± 0.08^{a}	3.83 ± 0.41b	0.00	0.00	0.09
16:1n-5	0.13 ± 0.04^{b}	0.07 ± 0.02^{b}	0.16 ± 0.04^{b}	0.31 ± 0.07^{a}	0.01	0.19	0.01
16:2n-4	0.27 ± 0.02	0.16 ± 0.05	0.39 ± 0.06	0.34 ± 0.12	0.03	0.18	0.65
17:0	0.24 ± 0.01^{ab}	0.13 ± 0.02^{b}	0.35 ± 0.01^{a}	0.28 ± 0.08^{a}	0.01	0.02	0.55
16:3n-4	0.26 ± 0.01	0.18 ± 0.01	0.31 ± 0.01	0.56 ± 0.28	0.08	0.43	0.17

16:3n-3	0.15 ± 0.01	0.11 ± 0.01	0.18 ± 0.01	0.42 ± 0.21	0.08	0.25	0.13
16:3n-1	0.13 ± 0.01	0.10 ± 0.01	0.17 ± 0.04	0.39 ± 0.23	0.11	0.30	0.20
16:4n-3	0.18 ± 0.01	0.14 ± 0.02	0.27 ± 0.04	0.46 ± 0.23	0.06	0.39	0.23
18:0	3.94 ± 0.42	3.60 ± 0.07	4.78 ± 0.66	3.63 ± 0.72	0.25	0.08	0.28
18:1n-9	16.16 ± 2.03	26.15 ± 1.74	16.79 ± 1.62	19.34 ± 5.01	0.19	0.02	0.13
18:1n-7	2.84 ± 0.24	2.95 ± 0.24	3.18 ± 0.16	5.03 ± 1.35	0.06	0.11	0.14
18:1n-5	0.19 ± 0.01	0.14 ± 0.02	0.23 ± 0.01	0.82 ± 0.74	0.17	0.28	0.22
18:2n-9	0.11 ± 0.01^{b}	0.09 ± 0.02^{b}	0.22 ± 0.07^{ab}	0.37 ± 0.13^{a}	0.01	0.24	0.15
18:2n-6	5.49 ± 1.03^{b}	10.59 ± 0.41^{a}	6.50 ± 1.12^{b}	8.11 ± 1.53^{ab}	0.34	0.00	0.05
18:2n-4	0.21 ± 0.00^{ab}	0.11 ± 0.01^{b}	0.25 ± 0.03^{ab}	0.38 ± 0.13^{a}	0.02	0.74	0.05
18:3n-6	0.23 ± 0.01	0.19 ± 0.04	0.37 ± 0.02	0.79 ± 0.42	0.06	0.27	0.19
18:3n-4	0.23 ± 0.05^{ab}	0.12 ± 0.02^{b}	0.21 ± 0.03^{ab}	0.42 ± 0.13^{a}	0.03	0.32	0.02
18:3n-3	1.21 ± 0.35^{b}	2.73 ± 0.34^{a}	1.34 ± 0.16^{b}	2.23 ± 0.38^{ab}	0.43	0.00	0.20
18:4n-3	0.82 ± 0.11	0.48 ± 0.08	0.94 ± 0.13	1.10 ± 0.43	0.07	0.62	0.19
18:4n-1	0.19 ± 0.01	0.08 ± 0.01	0.19 ± 0.03	0.38 ± 0.32	0.24	0.73	0.26
20:0	0.20 ± 0.02	0.16 ± 0.03	0.19 ± 0.03	0.51 ± 0.31	0.20	0.27	0.18
20:1n-9	0.27 ± 0.02	0.22 ± 0.03	0.24 ± 0.01	0.35 ± 0.21	0.55	0.67	0.35
20:1n-7	1.15 ± 0.06	1.37 ± 0.25	1.00 ± 0.15	0.88 ± 0.36	0.11	0.78	0.37
20:1n-5	0.25 ± 0.04	0.17 ± 0.03	0.29 ± 0.07	0.40 ± 0.33	0.31	0.87	0.49
20:2n-9	0.10 ± 0.04	0.07 ± 0.01	0.16 ± 0.01	0.45 ± 0.41	0.20	0.43	0.33
20:2n-6	0.31 ± 0.01	0.42 ± 0.05	0.33 ± 0.02	0.59 ± 0.32	0.46	0.17	0.56
20:3n-9	0.07 ± 0.01	0.04 ± 0.01	0.09 ± 0.03	0.24 ± 0.13	0.05	0.21	0.10
20:3n-6	0.20 ± 0.03	0.16 ± 0.01	0.23 ± 0.03	0.80 ± 0.61	0.19	0.29	0.23
20:4n-6	1.35 ± 0.04	0.79 ± 0.05	1.25 ± 0.22	1.00 ± 0.38	0.74	0.04	0.34
20:3n-3	0.25 ± 0.04	0.29 ± 0.05	0.28 ± 0.01	0.58 ± 0.36	0.28	0.25	0.39
20:4n-3	0.87 ± 0.01	0.60 ± 0.08	0.69 ± 0.03	0.64 ± 0.27	0.55	0.19	0.34
20:5n-3	9.75 ± 0.58	5.75 ± 0.36	7.65 ± 0.45	6.00 ± 3.00	0.45	0.05	0.34
22:1n-11	0.51 ± 0.11	0.44 ± 0.06	0.46 ± 0.02	0.73 ± 0.66	0.65	0.70	0.52

22:1n-9	0.29 ± 0.09	0.21 ± 0.03	0.26 ± 0.09	0.71 ± 0.82	0.48	0.56	0.42
22:4n-6	0.18 ± 0.01^{ab}	0.08 ± 0.02^{b}	0.33 ± 0.04^{ab}	0.85 ± 0.38^{a}	0.02	0.19	0.07
22:5n-6	0.43 ± 0.04	0.24 ± 0.03	0.58 ± 0.01	0.90 ± 0.53	0.09	0.74	0.25
22:5n-3	3.75 ± 0.17^{a}	2.79 ± 0.07^{ab}	2.17 ± 0.10^{b}	2.14 ± 0.56^{b}	0.00	0.06	0.08
22:6n-3	29.35 ± 3.78^{a}	21.75 ± 2.29^{b}	16.83 ± 0.81^{bc}	13.52 ± 0.83^{c}	0.00	0.01	0.17
Σ Saturates	18.16±0.16^{b}	17.34±1.11^{b}	29.34±1.56^{a}	23.37±3.18^{ab}	0.00	0.04	0.10
Σ Monoenes	25.62±2.83	34.55±2.15	28.66±1.68	32.73±4.10	0.76	0.02	0.26
Σ n-3	46.32±3.78^{a}	34.63±1.50^{b}	30.34±1.17^{b}	27.08±3.98^{b}	0.00	0.01	0.07
Σ n-6	8.17±0.92^{b}	12.47±0.44^{a}	9.57±0.85^{ab}	13.05±1.98^{a}	0.28	0.00	0.64
Σ n-3 HUFA	43.97±4.24^{a}	31.18±1.90^{b}	27.62±1.40^{b}	22.87±3.80^{b}	0.00	0.00	0.09
DHA/EPA	3.01±0.21	3.81±0.65	2.20±0.03	2.56±0.92	0.05	0.22	0.62
DHA/ARA	21.71±2.11^{ab}	27.60±1.20^{a}	13.71±1.76^{b}	14.84±5.28^{b}	0.00	0.15	0.31
n-3/n-6	5.73±1.10^{a}	2.78±0.19^{b}	3.19±0.40^{b}	2.14±0.63^{b}	0.01	0.00	0.05

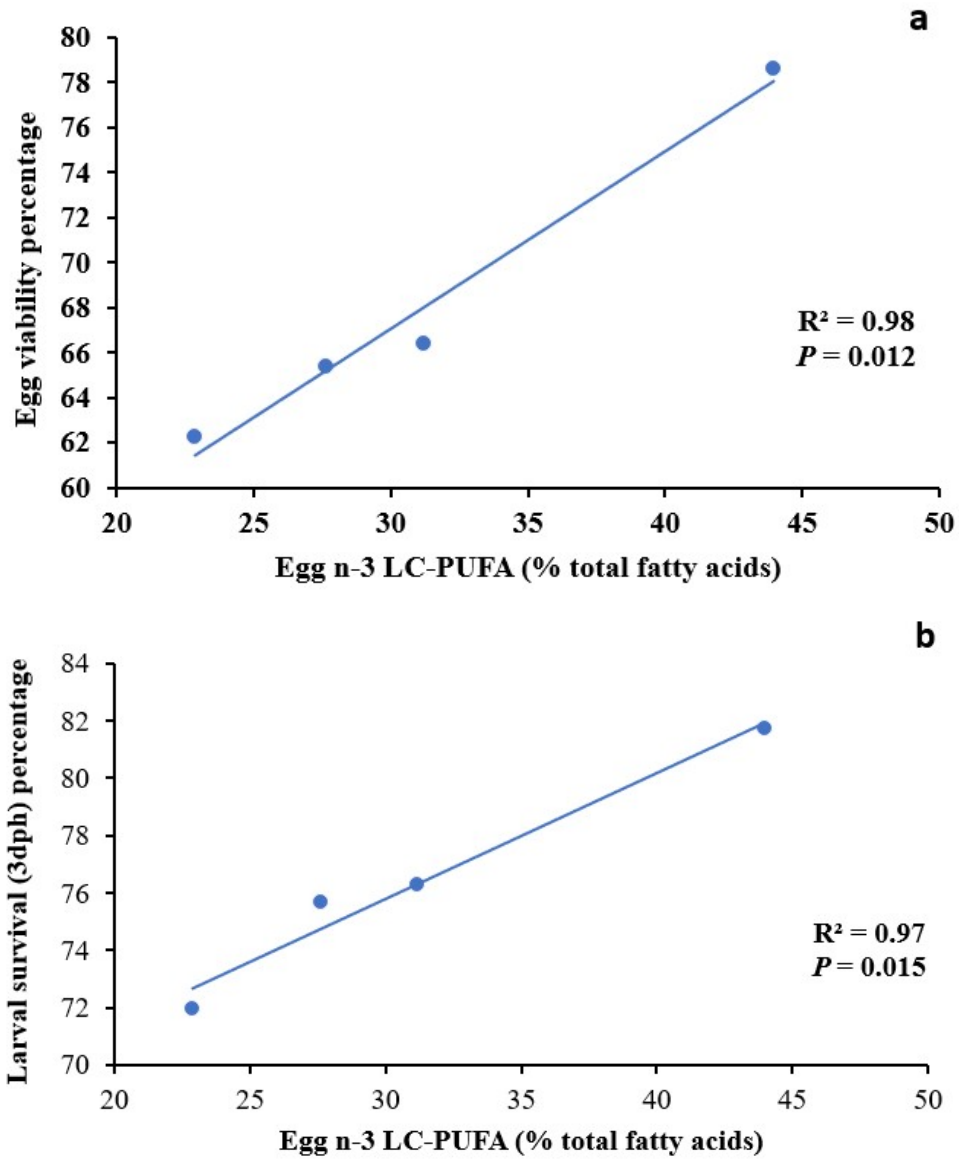

Figure 4-9. Effect of eggs n-3 LC-PUFA contents (one month after experimental diet feeding) and egg viability (a) or larval survival at 3 dph (b) (n=4) measured over the whole Phase III.

4.3.3.5 Molecular studies

The study of absolute *fads2* expression (mRNA copies/µl) in eggs from the different broodstock showed values that in average were 3 times higher for broodstock fed RO diet than for those fed FO (Figure 4-10). However, due to the large standard deviations, there were not significant (P>0.05) differences in *fads2* expression according to both the one-way or two-way ANOVA analysis.

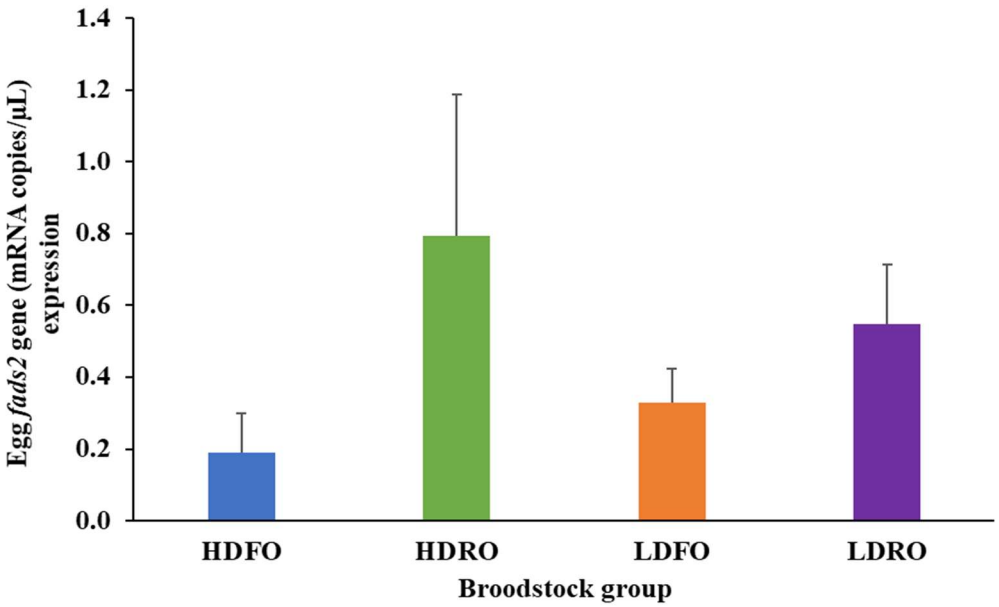

Figure 4-10. Expression of *fads2* in eggs (n=3) of gilthead seabream broodstock of high (HD) or low (LD) *fads2* expression fed with either FO or RO experimental diets at the end of Phase-III.

4.4 Discussion

Marine fish have a limited ability to synthesise LC-PUFAs, due to insufficient expression of key genes such as *fads2* and the inhibition of desaturase enzymes by dietary LC-PUFA (Izquierdo, 2005; Izquierdo et al., 2008; Seiliez et al., 2003, 2001; Tocher, 2015). Therefore, large FO replacement by VO in broodstock diets may lead to extremely low dietary LC-PUFA levels and markedly reduce broodstock performance (Fernández-Palacios, H., Norberg, B., Izquierdo, M., Hamre, 2011; Izquierdo et al., 2015). Optimizing FO replacement by VO is desired to induce nutritional programming in the offspring for a better use of low FM and low FO diets (Izquierdo et al., 2015; Turkmen et al., 2017a). Although, it has been found that there are large variations in the *fads2* expression showed by different gilthead seabream individuals, there is little information on its relation to fish reproductive performance (Turkmen et al., 2020). The present study thus aimed to determine the reproductive success of gilthead seabream broodstock with different bloods *fads2* expression fed diets containing different types of oils.

Plasma 17β-estradiol and 11-ketotestosterone levels showed respectively positive and negative correlations to the body weight of broodstock GSB, as is expected from a protandric hermaphrodite species that turns from male to female as it ages (Davis et al., 2007). The lack of relation between body weight and plasma testosterone levels as can be attributed to the fact that this hormone is an intermediate metabolite in most protandric fish (Godwin and Thomas,

1993). Broodstock body weight had no effect on *fads2* expression, in males or females, nor with any parameter regarding spawning quality. The higher level of expression of *fads2* (30% more in females than in the males) and the significant positive correlation between plasma 17β-estradiol levels and the *fads2* expression in females from the low *fads2* expression groups are noteworthy. These results are in agreement with the increased conversion of LNA to n-3 LC-PUFA promoted by oestrogen in women, claimed to be important to fulfil the essential fatty acid requirements of the foetus and the neonate (Burdge and Calder, 2005). In fish, n-3 LC-PUFA are also highly demanded during embryogenesis and larval development to sustain growth, normal development and neural and sensorial organs formation, with EPA and particularly DHA being the major fatty acids found in marine fish eggs and larvae (Izquierdo, 1996; Izquierdo et al., 2001). To our knowledge, this is the first study that finds a significant correlation between plasma oestrogen levels and *fads2* expression in blood cells of female fish, as observed in pregnant rats (Chalil et al., 2018; Childs et al., 2012). Indeed, treatments with 17β-estradiol have shown to increase *fads2* expression in female rats (Kitson et al., 2013). This higher *fads2* expression in females seems to be a response to the higher level of requirement of n-3 LC-PUFA fatty acid for the normal gonadal, oocyte and larval development through vitellogenetic processes in different teleosts (Callan et al., 2014; Harel et al., 1994; Izquierdo, 1996; Izquierdo et al., 2015, 2001; Mazorra et al., 2003). Interestingly, in females showing a very high expression of *fads2* such a strong correlation was not found, despite a general trend to increased *fads2* expression in females with higher oestrogen levels. These results suggest the influence of a different genetic or epigenetic background between both types of females showing very high or low *fads2* expression.

Very early, it was reported that stenohaline marine teleost have lower LC-PUFA bioconversion capacities compared to freshwater teleost (Kanazawa et al., 1979). More recent work found that freshwater fish to possess greater number of copies of Fads2, compared to marine fish and that the Fads2 was a key metabolic gene for overcoming some of the nutritional constraints associated with freshwater environment (Ishikawa et al., 2019). We observed that there was a very large variation in the individual *fads2* expression values, for males the maximum value being 47 times higher than the minimum one and for females 30 times, in agreement with the large variation found in our previous studies (Turkmen et al., 2020). From the 215 broodfish analysed for *fads2* expression, about 10% of the population had *fads2* expression values that were higher than 5 mRNA copies μL^{-1}. In humans, *FADS2* expression and delta-6 desaturase activity is linked to different *FADS2* genotypes and, in turn to lower

DNA methylation in specific CpG sites of the FADS2 promoter (Howard et al., 2014). In agreement with human studies, in gilthead seabream, there is an increased methylation of specific CpG sites in the promoter region of this gene in offspring from broodstock with low *fads2* expression (Turkmen et al., 2019). Moreover, LC-PUFA biosynthesis ability is affected by single nucleotide polymorphisms (SNPs) in the fatty acid desaturases (Schaeffer et al., 2006; Xie and Innis, 2008). Further studies are under way to elucidate the genetic and epigenetic mechanisms regulating *fads2* expression and LC-PUFA biosynthesis in gilthead seabream.

The improved reproductive performance in terms of sperm and egg quality in as observed here in broodstock with high blood *fads2* expression clearly reflects the importance of the end product(s) of *fads2* in fish reproduction. Sperm quality, in terms of motility and concentration, has been found to depend on its content in n-3 LC-PUFA, particularly DHA, in gilthead seabream after cryopreservation (Cabrita et al., 2005), as well as in rainbow trout (*Oncorhynchus mykiss*) (Labbe et al., 1995), European seabass (*Dicentrarchus labrax*) (Asturiano et al., 2001), Senegalese sole (*Solea senegalensis*) (Beirão et al., 2015) or European eel (*Anguilla Anguilla*) (Butts et al., 2015) fed different amounts n-3 LC-PUFA. Therefore, a higher *fads2* expression in gilthead seabream broodstock would allow a higher n-3 LC-PUFA synthesis to promote a higher incorporation of these fatty acids into sperm that would have improved sperm motility, affecting in turn egg viability. Besides, testis fatty acids profile may also affect seminal plasma, which maintains sperm cells in a quiescent state required to achieve motility (Gilroy and Litvak, 2019; Labbe et al., 1995). Finally, mice and human sperm also have high contents in DHA, which also acts as a precursor of very long chain LC-PUFAs with 26-32 carbons that form sphingolipids in spermatozoa and have been related to sperm quality (Stroud et al., 2009).

As regards seabream females where those with a higher *fads2* expression exhibited 20% higher fecundity in terms of eggs and larvae produced per kg female per spawn, this can also be linked to the production of n-3 LC-PUFA. Indeed, the DHA content in the eggs was effectively increased in females with a high *fads2* expression, denoting an efficient n-3 LC-PUFA biosynthesis in these females in comparison to those with low *fads2* expression, regardless of the diet fed. Interestingly, among the different LC-PUFA, only those from the n-3 series and with 22 carbons were significantly increased, despite the higher dietary content of 18:2n-6 (5.5-10.6% total fatty acid, TFA) in comparison to 18:3n-3 (1.2-2.7% TFA). Therefore, n-3/n-6 and DHA/ARA ratios were increased and 22:4n-6 reduced in females with a high *fads2* expression, suggesting the preference of the enzymatic complexes involved in fatty acid

deposition in eggs for the n-3 LC-PUFA. The higher DHA/EPA and 22:5n-3 (n-3 DPA) contents in the eggs also suggest the activation of the Sprecher pathway, since fish Fads2 have a Δ6 desaturase activity on 24:5n-3, produced by elongation from 22:5n-3, to synthesise DHA after beta-oxidation from 24:6n-3 (Oboh et al., 2017). Such high DHA and n-3 DPA contents in the egg may also be related to an increased mobilization of DHA to ovaries caused by 17β-estradiol, to a lower beta-oxidation of these fatty acids or to morphological changes in the ovaries. In turn, DHA increase in females would lead to an increased production of docosanoids, which play an important role in the induction of oocyte maturation (Sorbera et al., 2001), improving fecundity in terms of eggs produced. Our observation of a direct positive correlation between n-3 LC-PUFA contents in the egg and egg viability and larval survival confirms that these fatty acids are determinant of embryo and larval development (Callan et al., 2014; Harel et al., 1994; Izquierdo et al., 2001; Mazorra et al., 2003).

That the dietary fatty acid profile is reflected in tissue fatty acid profile is well established and our data show that broodstock fed with a diet rich in rapeseed oil led to increased levels of 18C fatty acids and relatively reduced levels of LC-PUFAs in egg lipids. These changes were however mild, where the 18C fatty acids increased by an average 60% in comparison to those from broodstock fed FO and the LC-PUFA were reduced by 30%, while in the RO diet these values were 200% and 20%, respectively in comparison to fish oil-based diet. This lower accumulation of 18C fatty acids together with the relatively lower reduction in LC-PUFA in the eggs, in comparison to the diet, suggests an increased LC-PUFA biosynthesis ability in conformity with the trend for an up-regulation of *fads2* expression found in the eggs of broodstock fed RO.

Broodstock diets replacement of FO by VO, namely reduction of dietary LC-PUFA and increase in 18:2n-6 and 18:3n-3, allows the conditioning of gilthead seabream offspring to produce juveniles which can potentially utilize low FM and FO diets better and grow fast (Izquierdo et al., 2015; Turkmen et al., 2017b,2019, 2020). Total replacement of FO by vegetable oils markedly reduces reproductive performance of gilthead seabream (Izquierdo et al., 2015; Turkmen et al., 2020), since LC-PUFA are essential for reproduction of this species (Fernández-Palacios et al., 1995; Fernández-Palacios et al., 2011). The partial FO replacement by RO as done here, giving dietary levels for LA+ALA: n-3 LC-PUFA ratio levels of 16:1:16.3 did not negatively affect sperm quality or spawning quality parameters. These results suggest that the LC-PUFA levels in diet RO were able to match the minimum requirements for gilthead seabream broodstock, in agreement with previous studies (Fernández-Palacios et al., 1995;

Fernández-Palacios et al., 2011). Thus, the ARA and EPA contents in diet RO were similar and those of DHA even higher than the optimum dietary levels determined for gilthead seabream broodstock (Fernández-Palacios et al., 1995). The levels of these LC-PUFA in the present study were also higher than those in broodstock diets used to induce nutritional programming in gilthead seabream, which caused a reduction in all spawning quality parameters (Izquierdo et al., 2015; Turkmen et al., 2020).

4.5 Conclusion

In summary, the results showed that blood *fads2* expression in gilthead seabream broodstock females, which tended to be higher than in males, was positively related to plasma oestrogen levels. Moreover, broodstock with high blood *fads2* expression showed a better reproductive performance, in terms of fecundity and sperm and egg quality, which was correlated with female *fads2* expression. Besides, the present study has demonstrated that it is feasible to reduce ARA, EPA and DHA down to 0.4, 6.6 and 8.4% of total fatty acids, respectively, without affecting spawning quality, in broodstock diets designed to induce nutritional programming effects in the offspring. Further studies are being conducted to test the offspring with low FM and FO diets along life span.

Authors' contribution

Conceived and designed the experiment: MI SF ST SK. Broodstock selection and *fads2* measurement: MI SF ST HX JMA. Diet formulation and preparation: MI SF RF GR. Plasma steroid hormone estimation: SF AG. Sperm quality evaluation: SF ST HX. Egg and larval quality evaluation: SF MI SK ST. Biochemical and molecular analysis: SF ST HX JMF. Analysed the data: SF MI SK AG. Wrote the paper: SF MI SK.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research, innovation program under grant agreement No 727610 (PerformFISH project). This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein. The first author gratefully acknowledges the financial assistance provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India and study leave by the Director, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India for the doctoral study.

CHAPTER 5

animals

Impact factor: 2.752

Influence of Genetic Selection for Growth and Broodstock Diet n-3 LC-PUFA Levels on Reproductive Performance of Gilthead Seabream, *Sparus aurata*

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Academic Editor: Jorge Galindo-Villegas

Animals **2021**, *11*(2), 519; https://doi.org/10.3390/ani11020519

Chapter 5

Abstract

Genetic selection in gilthead seabream (GSB), *Sparus aurata* has been undertaken to improve the growth, feed efficiency, fillet quality, skeletal deformities and disease resistance but no study is available to delineate the effect of genetic selection for growth trait on GSB reproductive performance under mass spawning condition. In this study, high growth (HG) or low growth (LG) GSB broodstock were selected to evaluate the sex steroid hormones, sperm, egg quality and reproductive performance under different feeding regime of commercial diet or experimental broodstock diet containing either fish oil (FO) or vegetable oil (VO) based diet. Under the commercial diet feeding phase, broodstock selected for either high growth or low growth did not show any significant changes in the egg production per kg female, whereas egg viability percentage was positively (P=0.014) improved by the high growth trait broodstock group. The experimental diet feeding results revealed that both growth trait and dietary fatty acid composition influenced the reproductive performance of GSB broodstock. In the experimental diet feeding phase, we observed high growth trait GSB male produced higher number of sperm cells (P<0.001) and also shown higher sperm motility (P=0.048) percentage. The egg and larval production per spawn per kg female were significantly improved by the broodstock selected for high growth trait and fed with fish oil based diet. This present study results signifies that gilthead seabream broodstock selected on growth trait could have positive role in improvement of sperm and egg quality to produce viable progeny.

Keywords: broodstock nutrition, fatty acids, nutritional programming, spawning quality

5.1 Introduction

As one of the major species under aquaculture in the Mediterranean region, the gilthead seabream (GSB) (*Sparus aurata*) is subject to selective breeding programmes (Antonello et al., 2009; Chavanne et al., 2016; Janssen et al., 2017; Knibb et al., 1997; Navarro et al., 2009). Selective breeding can be practiced following different methods, from the simplest mass selection to index selection and marker-assisted selection for production of offspring with desired traits. As mating system, all selection methods mainly use mass spawning in order to produce large quantities of the progeny required for selection process (Brown et al., 2005; Chavanne et al., 2016; Janssen et al., 2017). Conventional mass spawning has certain limitations for selection programs, since it may not be possible to identify the individual offspring's parents. To overcome this issue, microsatellite markers are used as a powerful tool to reconstruct the genealogy among offspring and breeders (Batargias, 1999; Lee-Montero et al., 2013; Navarro et al., 2009).

Broodstock selection in fish has been based on somatic growth (Fernandes et al., 2017; Navarro et al., 2009; Vu et al., 2019; Ye et al., 2017), feed efficiency (Besson et al., 2019; Callet et al., 2017; De Verdal et al., 2018), disease resistance (Li et al., 2020; Palaiokostas et al., 2016; Wiens et al., 2018), deformities (García-Celdrán et al., 2016; Negrín-Báez et al., 2015), fillet yield (Garcia et al., 2017; Schlicht et al., 2019; Vandeputte et al., 2019) or fillet fatty acid composition (Horn et al., 2020, 2018; Prchal et al., 2018). Selective breeding programs in gilthead seabream have also addressed improved growth performance, disease resistance and carcass quality (Afonso et al., 2012). Assessment of parental contributions in fast- and slow-growing progenies of gilthead seabream has been also assessed using a multiplex PCR (Borrell et al., 2011).

In Europe, between 31-44% of gilthead seabream seed comes from breeder selection processes (Chavanne et al., 2016), mainly for growth performance and morphology as selected traits (Janssen et al., 2017), and genetic improvement per generation of 5-29 % (Brown et al., 2005; Knibb et al., 1997). In Spain, seabream broodstock selection by Best linear unbiased prediction (BLUP) methodology has been based on somatic growth and morphology in commercial hatcheries (Fernandes et al., 2017), as well as on growth, carcass, flesh and fish quality and disease resistance on a series of jointly projects from commercial and public research hatcheries, known as PROGENSA (Afonso et al., 2012; García-Celdrán et al., 2016; Lee-Montero et al., 2013; Navarro et al., 2009; Negrín-Báez et al., 2015). In those projects, a weak association was found between families selected for growth (low and high Estimated Breeding Values (EBV)) and the type of diet (fish or plant ingredients based diet), denoting

that selection for faster growth is linked with different growth trajectories and a high diet flexibility and intestinal plasticity (Perera et al., 2019). However, the possible impact of genetic selection for somatic growth on reproductive performance of gilthead seabream is not well documented.

Being gilthead seabream a multi-batch spawner, egg quality very much relies on the continuous intake of nutrients to complete vitellogenesis during the whole spawning season. Therefore, adequate amounts of essential LC-PUFA must be provided in broodstock diets for the proper gonadal and embryonic development in this species (Fernández-Palacios et al., 1995). Given the limited availability of marine oils rich in LC-PUFA, recent work has also analysed the effects of diets containing alternative lipid sources such as microalgae (Atalah et al., 2007; Kissinger et al., 2016; Sarker et al., 2016; Sørensen et al., 2016) or krill oil (Betancor et al., 2012; Saleh et al., 2015), besides terrestrial vegetable oils (VO). It has been shown that partial replacement of fish oil by VO does not affect the spawning quality in gilthead seabream (Ferosekhan et al., 2020; Turkmen et al., 2020; Xu et al., 2019). In these studies, expression of the fatty acid desaturase 2 gene (*fads2*) was used as a potential biomarker. Gilthead seabream broodstock selected for high *fads2* gene expression exhibited improved egg and larval quality, even when fed with a low fish oil diet (Ferosekhan et al., 2020; Turkmen et al., 2020; Xu et al., 2019). Thus, high *fads2* broodstock fed relatively high amounts of linoleic (LA) and linolenic (ALA) acids showed an increased biosynthesis of n-3 LC-PUFA (Ferosekhan et al., 2020), and the subsequent nutritional programming improved the utilisation of low fish meal (FM) and fish oil (FO) diets by the progeny (Turkmen et al., 2019; Xu et al., 2019). This nutritional programming effect of feeding broodstock with low FO diets persisted in the progeny even up to the 16 months old juveniles (Turkmen et al., 2017b), and all the previous studies were based on the fatty acyl desaturase (*fads2*) as the potential biomarker to evaluate the reproductive performance of GSB. However, this present study was evaluated to ascertain the broodstock selection for growth trait (HG or LG) and fed with either FO or VO on the reproductive performance of GSB.

Therefore, the present study was conducted with gilthead seabream broodstock to ascertain the effect of genetic selection for high or low growth on reproductive performance and to determine the effect of selection for growth on improving the utilisation of vegetable oil-based diets under mass spawning programme. For that purpose, gilthead seabream broodstock selected for high (HG) or low (LG) growth were fed with two diets containing different lipid sources and based on fish oil (FO) or vegetable oil (rapeseed and linseed oil) along 3 months of the spawning season. The effect of the broodstock selection and the

broodstock diet on the seabream reproductive performance, plasma sex hormones levels and egg biochemical and fatty acid composition were studied under a two-way ANOVA design.

5.2 Materials and methods

5.2.1 Ethical statement

The study was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at Aquaculture Research Group (GIA) of ECOAQUA Institute, University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain. All experimentation performed at the (ULPGC) was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: 007/2012 CEBA ULPGC).

5.2.2 Experimental broodstock

The gilthead seabream broodstock used originated from third generation of selection under the PROGENSA (Spanish National Breeding Program) project (Afonso et al., 2012). Two broodstock groups expressing either high growth (HG), or low growth (LG), selected by BLUP methodology with VCE-v 6.0 software (Neumaier and Groeneveld, 1998), were used for the assessment of reproductive performance in mass spawning. HG and LG trait broodstock were individually marked with PIT tags (EID Iberica SA-TROVAN, Madrid, Spain) and maintained separately for mass spawning in four tanks (10 m^3) at the facilities of ECOAQUA institute (ULPGC, Canary Islands, Spain). All the tanks were supplied with seawater (37 g l^{-1} salinity, 17.8-19.0 °C) at a water exchange of 600% daily and maintained under natural photoperiod. The four broodstock groups were maintained separately for the whole reproductive season as HG (tanks 1 and 2) and LG (tanks 3 and 4), with an average biomass of 49 kg/tank and an average ratio female/male biomass of 1.5 (Table 5-1). Each group was formed by around 50 fish of 1 kg. Male body weight and female weight and size were larger in HG than in LG broodstock (Table 5-1).

Table 5-1. Description of gilthead seabream broodstock selected for mass spawning experiment

Broodstock details	HGFO	HGVO	LGFO	LGVO	One-way ANOVA *P* value
Broodstock density/tank					
Male (n)	32	33	27	27	
Female (n)	18	18	22	22	
Total (n)	50	51	49	49	

Broodstock biometry					
Male length (cm)	35.0±2.7	35.3±2.2	33.8±3.1	34.2±3.0	0.135
Male weight (kg)	1.0±0.3^{ab}	1.1±0.2^{a}	0.9±0.2^{b}	0.9±0.3^{ab}	0.015
Female length (cm)	35.8±4.3^{a}	35.2±3.2^{a}	32.3±1.5^{b}	32.8±1.8^{b}	< 0.001
Female weight (kg)	1.2±0.5^{a}	1.2±0.6^{a}	0.8±0.1^{b}	0.8±0.1^{b}	< 0.001
Male biomass (kg)	33	35	24	25	
Female biomass (kg)	22	21	17	18	
Total biomass (kg)	55	57	41	43	
Female/male biomass	1.5	1.7	1.4	1.4	

5.2.3 Phase I: Evaluation of spawning quality before feeding the experimental diet

The selected males and females from LG and HG broodstock groups were assessed for the spawning quality. At the beginning of the spawning season, from 20 December 2018 to 23 January 2019 brood fish were fed with a commercial diet (Europa Turbot 18, Skretting, Burgos, Spain) to ensure that there were no significant differences in the spawning quality among brood fish from the same selection group (HG or LG). Samples for analysis of sex steroid hormones and sperm quality were collected and analysis procedure described in below sections. Liver and gonad were excised and weighed for the determination of hepato-somatic index (HSI), and gonado-somatic index (GSI), respectively. Both these body indices were calculated as a percentage of organ to the whole-body weight of individual broodfish. For the evaluation of spawning quality, the spontaneously spawned eggs from each broodstock group were collected six times per week. Eggs were also collected at the end of the feeding period and kept at -80ºC until biochemical analysis.

5.2.4 Phase II: Evaluation of mass spawning quality after feeding the experimental diets

The experimental broodstock feeds were formulated to be iso-proteic and iso-lipidic with either fish oil (FO diet) or a mixture of vegetable oils (VO diet, containing rapeseed and linseed oil) as the lipid source and were produced by Skretting ARC, Stavanger, Norway (Table 5-2). Compared to the FO diet, the VO diet had higher levels of 18:2n-6 and 18:3n-3 fatty acids and reduced levels of saturated, monoenoic and n−3 LC-PUFA (20:5n−3; eicosapentaenoic acid, EPA and 22:6n−3; docosahexaenoic acid, DHA) (Table 5-3). The broodstock treatment groups were assigned as follows: HGFO, HGVO, LGFO and LGVO. Both the HG and LG brood fish groups were fed with one of the two diets at the rate of 1% body weight, twice a day (9:00 and 14:00 h), over a period of 3 months (24 January 2019 to 26 April 2019). Seawater temperature during broodstock spawning period was in the range of 18-22 °C (January-April, 2019) and fish were kept under natural photoperiod. Egg collection for spawning quality and biochemical composition followed the same protocol described in the commercial diet feeding

phase. Finally, after 3 months of feeding the two experimental diets, eggs were collected from all brood fish groups (HGFO, HGVO, LGFO and LGVO) and analysed for egg biochemical and fatty acid composition.

Table 5-2. Ingredients and proximate composition of the broodstock diets used for mass spawning study

Feed ingredients (%)	**FO diet**	**VO diet**
Fish meal, North-Atlantic	57.33	57.33
Krill meal	7.00	7.00
Squid meal	3.00	3.00
Wheat	21.99	21.99
Fish oil, South American	9.96	0.00
Rapeseed oil	0.00	8.45
Linseed oil	0.00	1.50
Vitamin-Mineral premix	0.50	0.50
Astaxanthin 10%	0.03	0.03
L-Histidine HCl	0.20	0.20
Proximate composition		
Crude protein (% dry matter, DM)	51.7	51.2
Crude lipid (% DM)	16.8	16.5
Ash (% DM)	10.6	10.4
Moisture (%)	8.4	8.9

Table 5-3. Fatty acid profiles (% total fatty acids) of the FO and VO broodstock diets

%TFA	**FO diet**	**VO diet**
14:0	3.44	2.17
14:1n-7	0.03	0.03
14:1n-5	0.11	0.08
15:0	0.30	0.16
15:1n-5	0.02	0.03
16:0 ISO	0.06	0.03
16:0	13.29	9.18
16:1n-7	5.12	2.38
16:1n-5	0.22	0.09
16:2n-6	0.01	0.00
16:2n-4	0.61	0.19
17:0	0.67	0.10
16:3n-4	0.13	0.15
16:3n-3	0.13	0.07
16:3n-1	0.08	0.05
16:4n-3	0.93	0.28
16:4n-1	0.03	0.02
18:0	3.54	2.53
18:1n-9	12.72	35.07
18:1n-7	2.93	2.79
18.1n-5	0.18	0.12

18:2n-9	0.04	0.02
18.2n-6	4.56	13.49
18:2n-4	0.23	0.05
18:3n-6	0.27	0.00
18:3n-4	0.15	0.07
18:3n-3	1.59	9.58
18.3n-1	0.03	0.01
18:4n-3	2.14	1.01
18:4n-1	0.14	0.03
20:0	0.52	0.49
20:1n-9	0.47	0.24
20:1n-7	3.88	3.19
20.1n-5	0.34	0.12
20:2n-9	0.05	0.02
20:2n-6	0.23	0.14
20:3n-9	0.09	0.04
20:3n-6	0.12	0.04
20:4n-6	1.00	0.28
20:3n-3	0.12	0.06
20:4n-3	0.67	0.21
20:5n-3	14.97	4.81
22:1n-11	5.20	3.20
22:1n-9	0.73	0.64
22:4n-6	0.14	0.06
22:5n-6	0.33	0.09
22:5n-3	1.91	0.36
22:6n-3	15.56	6.23
Total saturates	21.75	14.63
Total monoenes	31.93	47.98
Total n-3	38.01	22.62
Total n-6	6.659	14.10
Total n-9	14.09	36.03
Sum n-3 LC-PUFA	33.23	11.67
EPA/ARA	15.02	17.41
ARA/EPA	0.067	0.057
DHA/EPA	1.039	1.296
DHA/ARA	15.62	22.57
n-3/n-6	5.71	1.60

5.2.5 Plasma sex steroid hormones

All the broodstock were fasted overnight and anesthetized with clove oil (10 ppm clove oil:methanol (50:50) in sea water) to collect blood samples. Blood was taken from the caudal vein using sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 3.0 mL K3-EDTA tubes (L.P. Italiana, Milan, Italy). Whole blood samples were centrifuged at 3000 g for 10 min at 4 °C and plasma was separated and stored at -80 °C for sex steroid hormone analyses. Plasma sex steroids were measured by enzyme immunoassays (EIA) as described for European sea bass for testosterone (T) (Rodríguez et al., 2000), 11-ketotestosterone (11-KT) (Rodríguez et al., 2001) (and 17β-estradiol (E2) (Molés et al., 2008). Plasma steroids were

extracted with methanol and supernatants were dried and reconstituted in EIA buffer (potassium phosphate 0.1 M, pH 7.4 containing 0.01% sodium azide, 0.4 M NaCl, 0.001 M EDTA and 0.1% BSA). The assays were performed in 96-well plates coated with mouse anti-rabbit IgG monoclonal antibodies (Sigma-Aldrich, R-1008). Steroid standard curves (ranging from 0.0024-5.0 ng/ml for T; 0.0005-1.0 ng/ml for 11-KT and 0.039-80.0 ng/ml for E2; Sigma-Aldrich) or plasma samples were run in duplicate and added to the wells together with the corresponding. acetylcholinesterase (AChE) tracer: (T-AchE, 11-KT-AChE or E2-AChE; Cayman Chemical, Michigan, USA) and rabbit antiserum (anti-T, anti-11-KT or anti-E2), and incubated at 37ºC (E2) or 4ºC (T and 11-KT). Next, plates were rinsed, and color development was performed by addition of Ellman reagent. Optical density was read at 405 nm using a microplate reader (Bio-Rad 3550). The sensitivities of the assays (80% of binding) were 0,025 ng/ml for T, 0,0049 ng/ml for 11-KT and 0,30 ng/ml for E2. The inter-assay coefficients of variation at 50% of binding were 0.42% with a 0.90 slope for T, 32.6% with a 0.84 slope for 11-KT and 2.05% with a 0.83 slope for E2. The intra-assay coefficients of variation were 2.93% (n=4) for T, 5.65% (n = 6) for 11-KT and 0.83% (n=4) for E2. Sex steroid hormone concentration values are presented as mean ± SD.

5.2.6 Sperm quality

For sperm collection, fish were anesthetized as mentioned above and sperm was collected from the blot dried genital pore after a gentle abdominal massage to induce spermiation and taking care to avoid contamination with water, faeces or urine. The collected sperm was stored on ice until transferred to a 4 °C refrigerator. The sperm quality parameters that were evaluated included sperm concentration (number of spermatozoa x 10^9 ml^{-1}), spermatocrit %, sperm motility % (percentage of spermatozoa showing forward motility) and sperm motility duration (min). Sperm concentration was estimated after a 1000-fold dilution with sperm inactivation media using a Neubauer haematocytometer under 400x magnification. Sperm motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 µl of sperm with 50 µl of seawater (Cabrita et al., 2005; Felip et al., 2009).

5.2.7 Evaluation of egg and larval quality

The collected eggs were placed in 10 litre containers provided with aeration, from where 3 randomized 10 ml samples were taken and placed in a Bogorov chamber under the light microscope to calculate the total number of eggs (a fecundity parameter) and percentages

of fertilized and viable eggs. Egg viability was determined by observing the percentage of morphologically normal eggs after 1-day post fertilization (1 dpf) (Fernández-Palacios et al., 2011). Then, the viable eggs were individually placed in 96-well microtiter plates in two replicates filled with filtered and sterilized seawater. Eggs were incubated in a controlled temperature incubator at 19-21 °C, to estimate the percentage of hatching (2 dpf) and larval survival rates at 3 days post hatch (dph). From these values, other fecundity parameters were calculated, including total numbers of fertilized, viable, hatched and larvae produced per kg female (Fernández-Palacios et al., 1995).

5.2.8 Biochemical analysis

After feeding either the commercial diet or the experimental conditioning diets, egg samples were collected from all the broodstock groups and stored at -80°C for analysis of proximate and fatty acid composition. Moisture contents were obtained after drying the samples in an oven at 110°C for 24 h and then for 1 h until constant weight. Ash content was determined after incineration at 600°C for 16 h. Crude protein content was determined by measuring the N content (N×6·25) through automated Kjeldahl analysis (AOAC, 1995) and crude lipid extraction was carried out with chloroform: methanol (Folch et al., 1957). Fatty acids from total lipids were prepared by transmethylation and separated by gas chromatography (Izquierdo, 1989) and identified by comparison with previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

5.2.9 Statistical analysis

Data are reported as mean ± standard deviation. Data were compared statistically using the analysis of variance (ANOVA), at a significance level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene's tests, respectively (Sokal et al., 1979). Otherwise, an arcsin transformation was performed to attain normality. When arcsin-transformed data were not normally distributed, then Kruskall–Wallis non-parametric test was applied to the non-transformed data. An independent sample *t*-test was performed to compare sex steroid hormones, spawning quality, egg biochemical and fatty acid composition for commercial diet feeding phase to check the broodstock selection (HG or LG) effect. One way and two-way ANOVA were applied to the results of sex steroid hormones, sperm and egg and larval quality parameters (total eggs; fertilized eggs; viable eggs; hatched larvae; 3dph larvae per spawn per kg female and fertilization, egg viability, hatching and larval survival rates), egg biochemical and fatty acid composition of experimental diet

feeding phase to determine the combined effects of broodstock selection (HG or LG) and diet (FO or VO). Pearson's correlation coefficient and regression analysis were performed to check the relationship between spawning quality parameters. All data were analysed using the program IBM SPSS version 20 for Windows (IBM SPSS Inc.).

5.3 Results

5.3.1 Phase I: Evaluation of spawning quality before feeding the experimental diet

5.3.1.1 Plasma sex steroid hormones

Male and female plasma sex steroid hormone levels were not significantly different between HG or LG broodstock fed the same commercial diet (Table 5-4). The levels of plasma testosterone and 17β-estradiol were, respectively, 13 and 20% higher in HG than in LG female broodstock (Table 5-4). Plasma testosterone level was significantly higher in males than in females, being almost 2.9 and 3.3 folds higher in males than in females of HG or LG broodstock, respectively. Pearson's correlation analysis result revealed that there was no correlation between broodstock body weight and steroid sex hormone levels (Table 5-5). There was a strong positive correlation (r=0.852; *P*=0.001) between plasma testosterone and 11-ketotestosterone levels in males and, to a lower extent (r=0.500; *P*=0.05), between testosterone and 17β-estradiol levels in females. The GSI was significantly higher in HG males than in LG males (Table 5-4), whereas female GSI showed large deviations and no significant differences were found between HG and LG females for this parameter. No differences were found in HSI of males or females between HG and LG broodstock (Table 5-4), neither any relation between broodstock body weight and the foresaid parameters (Table 5-6).

Table 5-4. Hepatosomatic, gonadosomatic index, steroid sex hormone levels of gilthead seabream male or female broodstock of high (HG) or low growth (LG) groups before feeding the experimental diets. Male-HG (n=6); Male-LG (n=7); Female-HG (n=6) and Female-LG (n=5).

Broodstock Sex		High growth (HG)	Low growth (LG)	t-test (*P* value)
Male	Testosterone (ng/ml)	2.3±0.8	2.3±1.4	0.989
	11 Keto-testosterone (ng/ml)	0.2±0.1	0.4±0.3	0.243
	GSI%	7.8±2.5[a]	2.0±2.4[b]	0.001
	HSI%	1.0±0.2	1.5±0.9	0.211
Female	Testosterone (ng/ml)	0.8±0.2	0.7±0.4	0.649
	17β-estradiol (ng/ml)	1.0±0.3	0.8±0.3	0.337
	GSI%	6.3±9.7	5.1±4.8	0.805
	HSI%	1.7±0.4	1.4±0.4	0.236

Different superscripts in each row indicate significant differences among HG or LG broodfish groups ($P<0.05$, independent sample *t*-test).
GSI (%) (gonadosomatic index) = (Gonad weight, g/weight of fish, g) x 100; HSI (%) (hepatosomatic index) = (Liver weight, g/weight of fish, g) x 100

Table 5-5. Pearson's correlation coefficient of broodstock body weight, hepatosomatic, gonadosomatic index, steroid sex hormone levels of gilthead seabream male or female broodstock of high (HG) or low growth (LG) groups before feeding the experimental diets

Pearson's correlation	HSI %	GSI %	TST ng/ml	11KT ng/ml	E2 ng/ml
Broodstock body weight (g)	-0.15	0.26	0.22	-0.01	0.06
HSI %	-	-0.22	-0.511*	-0.466*	-0.04
GSI %	-0.22	-	-0.17	-0.22	0.01
TST (ng/ml)	-0.511*	-0.17	-	0.852**	0.500*
11KT (ng/ml)	-0.466*	-0.22	0.852**	-	0.24
E2 (ng/ml)	-0.04	0.01	0.500*	0.24	-

The symbol *, ** denotes significant differences as $P < 0.05$ or 0.01 level

Table 5-6. Regression relationship analysis between broodstock body weight and HSI, GSI, TST, 11KT and E2

Regression analysis	HSI %		GSI %		TST ng/ml		11KT ng/ml		E2 ng/ml	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Broodstock body weight	0.034	0.422	0.083	0.204	0.049	0.335	0.015	0.949	0.004	0.796

5.3.1.2 Evaluation of egg and larval quality

After one month of feeding the commercial diet at the beginning of the spawning season (December-January), there were no significant differences (P>0.05) in reproductive performance in terms of fecundity (expressed per spawn and per kg female) between HG or LG broodstock (Table 5-7). The number of viable eggs/spawn /kg female was 15% higher for HG than for LG broodstock, showing the lowest P value, but still not significantly different (Table 5-7). Accordingly, in terms of larval output, HG broodstock produced relatively higher (15%) number of 3 dph larvae per spawn per kg female than the LG broodstock (Table 5-7). Spawning quality parameters, in terms of percentage of fertilization, hatching and larval survival, were not significantly different (P>0.05) between HG and LG broodstock (Table 5-8). However, egg viability was significantly (P<0.001) higher for HG than for LG broodstock (Table 5-8).

Table 5-7. Reproductive performance (fecundity) of gilthead seabream broodstock selected for high (HG) or low (LG) growth before experimental diet feeding period (HG, n=56; LG, n=54).

Fecundity parameters	High growth (HG)	Low growth (LG)	t-test (*P* value)
Total number of eggs/spawn/kg female	21,534 ± 15024	20,549 ± 15430	0.735
Fertilized eggs/spawn/kg female	21,114 ± 14749	20,114 ± 15242	0.727
Viable eggs/spawn/kg female	16,141 ± 11079	13,689 ± 12319	0.275
Hatched larvae/spawn/kg female	15,872 ± 10389	13,744 ± 10773	0.340
Larval survival 3dph/spawn/kg female	12,644 ± 8804	10,762 ± 9560	0.334

Table 5-8. Relative spawning quality (%) in gilthead seabream broodstock selected for high (HG) or low (LG) growth before experimental diet feeding period (HG, n=56; LG, n=54).

Spawning quality parameters	High growth (HG)	Low growth (LG)	t-test (*P* value)
Fertilization %	96.4 ± 8.9	96.7 ± 4.5	0.617
Egg viability %	75.4 ± 16.6^{a}	64.6 ± 24^{b}	0.014
Hatching %	92.2 ± 7.7	91 ± 12.1	0.762
Larval survival (3dph) %	80.2 ± 14.3	78.4 ± 17.1	0.731

Means bearing different superscript letters differ significantly (P<0.05, Independent sample t-test).

5.3.1.3 Biochemical analysis

The biochemical (Table 5-9) and fatty acid composition (Table 5-10) of the eggs also did not show significant (P>0.05) differences between HG or LG broodstock fed with the commercial diet. The crude protein and crude lipid contents in eggs were found to be more than 68% and 18% in both HG or LG broodstock group (Table 5-9). The fatty acid profile of eggs from broodstock fed the commercial diet did not differ much between high or low growth broodstock (Table 5-10) and only 18:2n-9 (P=0.005) and 18:3n-3 (P=0.041) were respectively

slightly lower and higher in HG than in LG eggs. Although they were all fed the same diet over the one-month period, all the individual LC-PUFA including EPA, DHA, ARA and, particularly 20:3n-3 (P=0.085) were slightly higher in the eggs from broodstock selected for high growth (HG) than in those from LG group (Table 5-10). Consequently, the total contents in PUFA from n-3 or n-6 series were respectively 18% and 5% higher in the eggs from broodstock selected for high growth (HG), whereas, saturated and monounsaturated fatty acids tend to be lower than in those from LG group, nevertheless none of this tendencies was significant (P>0.05) (Table 5-10).

Table 5-9. Biochemical composition of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth before feeding broodstock experimental diets (HG, n=3; LG, n=3).

Biochemical composition	High growth (HG)	Low growth (LG)	t-test (*P* value)
Crude protein (% DM)	72.6 ± 5.3	68.7 ± 3.6	0.171
Crude lipid (% DM)	18.4 ± 7.9	23.5 ± 0.8	0.146
Ash (% DM)	2.7 ± 1.2	3.6 ± 0.3	0.148
Moisture (%)	88 ± 0.5	87.7 ± 0.3	0.213

Table 5-10. Fatty acid composition (% total fatty acids) of eggs of gilthead sea bream broodstock selected for high (HG) or low (LG) growth before feeding the experimental diets (HG, n=2; LG, n=2)

Fatty acid (%TFA)	High growth (HG)	Low growth (LG)	t-test (*P* value)
14:0	1.54±0.29	1.55±0.49	0.980
14:1n-7	0.01±0.01	0.01	0.391
14:1n-5	0.07±0.02	0.07±0.02	0.834
15:0	0.23±0.03	0.24±0.07	0.696
16:0 ISO	0.08±0.08	0.09±0.08	0.896
16:0	14.00±0.89	16.23±3.34	0.246
16:1n-7	3.10±0.58	3.71±0.53	0.175
16:1n-5	0.08±0.03	0.08±0.01	1.000
16:2n-4	0.15±0.02	0.14±0.01	0.267
17:0	0.14±0.01	0.14±0.02	0.628
16:3n-4	0.25±0.02	0.26±0.04	0.414
16:3n-3	0.08±0.01	0.09±0.02	0.304
16:3n-1	0.08±0.02	0.09±0.02	0.344
16:4n-3	0.07±0.02	0.07±0.02	0.613
18:0	3.80±0.50	4.45±1.05	0.320
18:1n-9	27.78±2.24	29.59±4.46	0.506
18:1n-7	2.71±0.39	2.80±0.45	0.770
18:1n-5	0.13±0.01	0.15±0.02	0.211
18:2n-9	0.19±0.02[b]	0.24±0.01[a]	0.005
18.2n-6	12.29±0.51	12.01±1.07	0.655
18:2n-4	0.13±0.01	0.13±0.01	1.000
18:3n-6	0.28±0.03	0.31±0.01	0.161
18:3n-4	0.16±0.03	0.15±0.04	0.704

18:3n-3	2.72±0.16[a]	2.41±0.18[b]	0.041
18:4n-3	0.47±0.05	0.38±0.11	0.217
18:4n-1	0.09±0.01	0.07±0.02	0.124
20:0	0.09±0.01	0.10±0.03	0.758
20:1n-9	0.16±0.03	0.16±0.03	0.824
20:1n-7	0.98±0.14	0.98±0.21	0.970
20.1n-5	0.12±0.02	0.12±0.03	0.879
20:2n-9	0.09±0.00	0.09±0.01	0.705
20:2n-6	0.47±0.04	0.45±0.07	0.681
20:3n-6	0.22±0.01	0.21±0.02	0.439
20:4n-6	0.68±0.03	0.64±0.08	0.418
20:3n-3	0.34±0.02	0.31±0.03	0.085
20:4n-3	0.73±0.06	0.61±0.15	0.182
20:5n-3	4.39±0.54	3.63±1.42	0.378
22:1n-11	0.19±0.02	0.20±0.05	0.620
22:1n-9	0.09±0.01	0.10±0.03	0.656
22:4n-6	0.08±0.01	0.07±0.01	0.320
22:5n-6	0.22±0.02	0.19±0.06	0.396
22:5n-3	2.30±0.34	1.78±0.78	0.289
22:6n-3	18.22±3.43	14.90±7.97	0.486
Total Saturates	19.80±1.20	22.69±4.56	0.265
Total Monoenes	35.43±2.74	37.96±5.62	0.459
Total n-3	29.31±4.29	24.19±10.52	0.418
Total n-6	14.22±0.51	13.87±1.09	0.574
Total n-9	28.34±2.26	30.20±4.53	0.500
Total n-3 LC-PUFA	25.98±4.30	21.24±10.31	0.443
EPA+DHA	22.61±3.92	18.53±9.38	0.467
ARA/EPA	0.16±0.03	0.20±0.06	0.307
EPA/ARA	6.50±0.99	5.54±1.65	0.359
DHA/ARA	27.01±5.62	22.50±10.19	0.476
DHA/EPA	4.14±0.42	3.91±0.68	0.596
DHA/DPA	7.89±0.39	8.06±1.02	0.775
n-3/n-6	2.07±0.37	1.79±0.86	0.577
n-6/n-3	0.49±0.09	0.69±0.34	0.346
20:2n-9/20:1n-9	0.57±0.08	0.60±0.07	0.579
18:3n-6/18:2n-6	0.02±0.00	0.03±0.01	0.182
20:3n-6/20:2n-6	0.47±0.05	0.47±0.08	0.959
18:4n-3/18:3n3	0.17±0.02	0.16±0.03	0.543
20:4n-3/20:3n-3	2.15±0.23	1.96±0.42	0.453

Means bearing different superscript letters differ significantly ($P<0.05$, Independent sample *t*-test).

5.3.2 Phase II: Evaluation of mass spawning quality after feeding the experimental diets

5.3.2.1 Plasma sex steroid hormones

One-way ANOVA results indicated that plasma testosterone levels in LGFO and LGVO males were significantly ($P=0.033$) higher than in HGVO broodstock (Table 5-11), whereas in females, LGFO and HGVO were lower in plasma testosterone ($P=0.004$) than HGFO females (Table 5-11). Besides, 17β-estradiol levels in the LGFO females were

significantly (P=0.002) lower than in LGVO and HGVO (Table 5-11). The two-way ANOVA analysis showed that male testosterone (P=0.027) and 11-keto-testosterone (P=0.029) levels were significantly higher in LG broodstock (Table 5-11), whereas female 17β-estradiol levels were significantly (P=0.001) higher in broodstock fed the VO diet (Table 5-11). Moreover, there was a significant (P=0.001) interaction between selection and diet in the female testosterone plasma levels that in HG broodstock were higher when fish was fed the FO diet, whereas the opposite trend could be found in LG broodstock (Table 5-11). Pearson's correlation coefficient analysis showed that male 11-ketotestosterone levels were significantly negatively (r=-0.504; P=0.05) correlated to broodstock body weight (Table 5-12), following a linear relation (Table 5-13), whereas female body weight did not show any relation to steroid hormone levels. There was also a significant positive correlation (r=0.699; P=0.002) between male testosterone and 11-ketotestosterone hormone levels.

7.3.2.2 Sperm quality

Regarding sperm quality, sperm from the broodstock selected for high growth showed a significantly (P<0.01) higher cell concentration (Table 5-14), regardless the diet fed, reflected in the strong effect of broodstock selection observed in the two-way ANOVA (Table 5-14). Whereas sperm viability was not affected by either broodstock selection or diet, sperm motility (95-98%) was significantly (P=0.048) increased in the HG broodstock. On the contrary, sperm motility duration was increased by feeding both HG and LG broodstock with the VO diet (P<0.001) (Table 5-14). Besides, the sperm motility was highly significantly correlated (r=0.635; P=0.015) to sperm concentration, and mildly correlated (r=0.521; P=0.056) to sperm viability (Table 5-15). Besides, sperm concentration was slightly correlated (r=-0.544; P=0.055) to plasma 11-ketotestosterone levels (Table 5-15) followed a linear regression (R^2=0.409; P=0.018) (Table 5-16). Also, sperm concentration followed a significant linear relationship with testosterone (R^2=0.305; P=0.050) (Table 5-16), whereas other sperm parameters were not influenced by the male steroid hormones.

Table 5-11. Steroid sex hormone levels of gilthead seabream male or female broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season (HGFO, n=5; HGVO, n=5; LGFO, n=5; LGVO, n=5).

Broodstock sex	Steroid sex hormone	Broodfish groups				One-way ANOVA	Two-way ANOVA (*P* values)		
		HGFO	HGVO	LGFO	LGVO	(*P* values)	Selection	Diet	S x D
Male	Testosterone (ng/ml)	2.2±0.2^{ab}	1.2±0.4^{b}	2.9±0.4^{a}	2.8±0.4^{a}	0.033	0.027	0.383	0.561
	11 Keto-testosterone (ng/ml)	0.2±0.01	0.04±0.01	0.1±0.04	0.1±0.01	0.085	0.029	0.256	0.616
Female	Testosterone (ng/ml)	2.5±0.4^{a}	1.1±0.2^{b}	1.1±0.1^{b}	1.6±0.2^{ab}	0.004	0.074	0.089	0.001
	17β-estradiol (ng/ml)	1.8±0.4^{ab}	2.8±0.4^{a}	0.8±0.1^{b}	2.6±0.3^{a}	0.002	0.075	0.001	0.285

Different superscripts in each row would indicate significant differences among broodfish groups for a given parameter (P<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 5-12. Pearson's correlation coefficient of broodstock body weight, steroid sex hormone levels of gilthead seabream male or female broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season.

Male broodstock	Body weight	Testosterone (ng/ml)	11 Keto-testosterone (ng/ml)
Body weight	-	-0.29	-0.504*
Testosterone (ng/ml)	-0.29	-	0.699**
11 Keto-testosterone (ng/ml)	-0.504*	0.699**	-
Female broodstock	**Body weight**	**Testosterone (ng/ml)**	**17β-estradiol (ng/ml)**
Body weight	-	-0.098	-0.052
Testosterone (ng/ml)	-0.098	-	-0.134
17β-estradiol (ng/ml)	-0.052	-0.134	-

The symbol *, ** denotes significant differences as $P < 0.05$ or 0.01 level

Table 5-13. Regression relationship analysis between broodstock body weight and steroid sex hormone levels of gilthead seabream male or female broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season.

Regression analysis	Testosterone (ng/ml)		11 Keto-testosterone (ng/ml)		17β-estradiol (ng/ml)	
	R^2	*P*	R^2	*P*	R^2	*P*
Male body weight	0.085	0.257	0.254	**0.039**	-	-
Female body weight	0.010	0.707	-	-	0.004	0.816

Table 5-14. Sperm quality of gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or VO diet over three months of reproductive season (HGFO, n=4; HGVO, n=4; LGFO, n=5; LGVO, n=5).

Sperm quality parameters	Broodfish groups				One-way ANOVA (*P* values)	Two-way ANOVA (*P* values)		
	HGFO	HGVO	LGFO	LGVO		Selection	Diet	S x D
Sperm concentration (10^9 cells/ml)	9.8±0.7[a]	10.3±0.9[a]	3.9±0.8[b]	5.4±1.6[b]	0.0001	<0.001	0.210	0.512
Sperm viability %	86.4±5.3	91.3±7.7	89.2±6.6	92.8±1.8	0.5012	0.507	0.202	0.847
Sperm motility %	95.0±3.5	98.8±1.8	75±15.5	91.0±5.2	0.0504	0.048	0.140	0.344
Sperm motility duration (Seconds)	751±26[b]	1287±314[a]	777±172[b]	1764±184[a]	<0.0001	0.047	<0.0001	0.070

Means bearing different superscript letters differ significantly (P<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 5-15. Pearson's correlation coefficient of steroid sex hormone levels and sperm quality of gilthead seabream male broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season.

Pearson's correlation		Sperm concentration (10^9 cells / ml)	Sperm viability %	Sperm motility %	Sperm motility duration (sec)
Testosterone (ng/ml)	r	-0.456	0.100	-0.250	-0.086
	P	0.117	0.744	0.411	0.780
11 Keto-testosterone (ng/ml)	r	-0.544	-0.162	-0.510	-0.259
	P	0.055	0.598	0.075	0.394
Sperm concentration (10^9 cells / ml)	r	-	0.053	0.635*	0.057
	P	-	0.858	0.015	0.848
Sperm viability %	r	0.053	-	0.411	0.521
	P	0.858	-	0.144	0.056
Sperm motility %	r	0.635*	0.411	-	0.450
	P	0.015	0.144	-	0.106
Sperm motility duration (sec)	r	0.057	0.521	0.450	-
	P	0.848	0.056	0.106	-

The symbol *, ** denotes significant differences as $P < 0.05$ or 0.01 level.

Table 5-16. Regression relationship analysis between steroid sex hormones and sperm quality of gilthead seabream male broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season.

Regression analysis	Sperm Concentration (10^9 cells / ml)		Sperm viability %		Sperm motility %		Sperm motility duration (sec)	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Testosterone (ng/ml)	0.305	0.050	0.010	0.744	0.062	0.411	0.07	0.780
11 Keto-testosterone (ng/ml)	0.409	0.018	0.026	0.598	0.261	0.075	0.067	0.394

5.3.2.3 Evaluation of egg and larval quality

Fecundity in terms of total number of eggs produced per spawn per kg female was significantly (P<0.001) highest for LGFO broodstock (Table 5-17). Therefore, the two-way ANOVA analysis denoted a significantly (P=0.009) higher number of eggs produced per spawn per kg female in broodstock fed the FO diet and selected for LG, denoting the interaction between selection and diet (P=0.005) (Table 5-17). The same results were also found for the number of fertilized eggs (Table 5-17). On the contrary, there was not a combined effect of selection and diet for the other fecundity parameters studied, which were all significantly (P<0.001) improved in broodstock fed the FO diet, regardless the selection group (Table 5-17). Thus, the number of viable eggs hatchlings and larvae, per spawn per kg female was significantly higher for HGFO and LGFO than for LGVO or HGVO broodfish (Table 5-17).

Regarding the relative spawning quality parameters, fertilization rates were significantly (P<0.001) highest in HGFO eggs and lowest in LGVO eggs (Table 5-18), the two-way ANOVA showing the strong improvement in this parameter of either selection of HG broodstock (P=0.019) or feeding FO diets (P<0.001). Egg viability rate was even more strongly affected by the broodstock selection (P=0.000) and broodstock diet (P<0.001) (Table 5-18). Thus, the egg viability rate was high (93%) in HGFO broodstock, followed by LGFO (63.7%), whereas both LGVO (55%) and HGVO (54%) broodfish produced a significantly (P<0.001) lower proportion of viable eggs (Table 5-18). The hatching and larval survival rates were found to be higher in FO diet fed broodstock irrespective of the selection criterion (Table 5-18). These findings clearly indicate that dietary fatty acids from FO source had strong positive influence on gilthead seabream broodstock reproductive performance and high growth selected broodstock had higher proportions of fertilized and viable eggs.

The estimated total number of eggs spawned by the total number of female per tank, or by single female, or by kg body weight females were estimated and also calculated the egg mass (g, wet weight) production per spawn per kg female. The results indicated that gilthead seabream female broodstock (kg female) produces 2.18 to 2.88 million eggs and 0.58 to 1.12 million larvae (3dph) in 3 months spawning period (January to April). It was also noticed that HGFO broodstock (18 females) group produced relatively higher number of larvae (24.55 million) in entire 3 months spawning season compared to other broodstock group. The larvae production per kg female was also relatively higher for HGFO broodstock (1.12 million). The

egg mass production per spawn per kg female was found to be 9.5 to 12 g (mean egg wet weight ≈ 350 mg) for gilthead seabream broodstock.

Pearson's correlation coefficient analysis showed that sperm viability had a strong positive correlation (r=0.997; *P*=0.003) to egg fertilization rate and egg viability (r=0.957; *P*=0.043). There was also a positive correlation between egg fertilization rate and egg viability (r=0.934; *P*=0.066), as well as between egg hatching rate (r=0.957; *P*=0.043) and larval survival rate (Table 5-19). We also found a significant linear relationship between sperm viability and fertilisation rate (R^2=0.995; *P*=0.003) and viability (R^2=0.915; *P*=0.043) percentage; sperm motility duration had a slight linear relation to egg fertilization rate (R^2=0.863; *P*=0.071) (Table 5-20).

Table 5-17. Reproductive performance (fecundity) of gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or VO diet over three months of reproductive season (HGFO, n=70; HGVO, n=67; LGFO, n=72; LGVO, n=72).

Fecundity parameters	Broodfish groups				One-way ANOVA (*P* values)	Two-way ANOVA (*P* values)		
	HGFO	HGVO	LGFO	LGVO		Selection	Diet	S x D
Total number of eggs/spawn/kg female	28142±10552^{b}	28451±10502^{b}	34478±11100^{a}	27145±12667^{b}	< 0.001	0.062	0.009	0.005
Fertilized eggs/spawn/kg female	27946±10493^{b}	28100±10479^{b}	34140±11083^{a}	26746±12643^{b}	< 0.001	0.072	0.007	0.005
Viable eggs/spawn/kg female	22853±9854^{a}	15326±6631^{b}	21868±9233^{a}	14767±8589^{b}	< 0.001	0.457	< 0.001	0.837
Hatched larvae/spawn/kg female	21546±9493^{a}	13407±6317^{b}	20351±8895^{a}	13650±8276^{b}	< 0.001	0.633	< 0.001	0.472
Larval survival 3dph/spawn/kg female	16333±9274^{a}	8707±5745^{b}	14793±7956^{a}	9100±7012^{b}	< 0.001	0.529	< 0.001	0.289

Means bearing different superscript letters differ significantly (P<0.05 by One-way ANOVA, Tukey Post-Hoc).

Table 5-18. Relative spawning quality (%) in gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or VO diet over three months of reproductive season (HGFO, n=70; HGVO, n=67; LGFO, n=72; LGVO, n=72).

Spawning quality parameters	Broodfish groups				One-way ANOVA (*P* values)	Two-way ANOVA (*P* values)		
	HGFO	HGVO	LGFO	LGVO		Selection	Diet	S x D
Fertilization %	99.4±1.7^{a}	98.7±2.3^{b}	99±1.8^{ab}	98.4±2.8^{b}	< 0.001	0.019	< 0.001	0.397
Egg viability %	81.3±15.6^{a}	54.3±13.6^{c}	63.7±18.1^{b}	54.9±15.7^{c}	< 0.001	0.000	< 0.001	0.000
Hatching %	93.6±9.7^{a}	87.2±13.4^{b}	92.6±9.4^{a}	90.6±10.7^{ab}	< 0.001	0.392	< 0.001	0.037
Larval survival (3dph) %	75.3±20.6^{a}	64.4±23.1^{b}	73.1±20.6^{ab}	67.1±22^{b}	0.003	0.863	< 0.001	0.210

Means bearing different superscript letters differ significantly (P<0.05 by One-way ANOVA, Tukey Post-Hoc).

Table 5-19. Pearson's correlation coefficient of sperm quality and egg quality of gilthead seabream broodstock of high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season

Pearson's correlation		Sperm concentration (10^9 cells/ml)	Sperm viability %	Sperm motility %	Sperm motility duration (sec)	Egg fertilization %	Egg viability %	Hatching %	Larval survival (3dph) %
Sperm concentration (10^9 cells/ml)	r	-	-0.268	0.884	-0.090	0.289	0.245	-0.380	-0.202
	P	-	0.732	0.116	0.910	0.711	0.755	0.620	0.798
Sperm viability %	r	-0.268	-	0.094	0.908	0.997**	0.957*	-0.710	-0.874
	P	0.732	-	0.906	0.092	0.003	0.043	0.290	0.126
Sperm motility %	r	0.884	0.094	-	0.358	-0.094	-0.009	-0.506	-0.453
	P	0.116	0.906	-	0.642	0.906	0.991	0.494	0.547
Sperm motility duration (sec)	r	-0.090	0.908	0.358	-	-0.929	-0.758	-0.592	-0.796
	P	0.910	0.092	0.642	-	0.071	0.242	0.408	0.204
Egg fertilization %	r	0.289	0.997**	-0.094	-0.929	-	0.934	0.669	0.849
	P	0.711	0.003	0.906	0.071	-	0.066	0.331	0.151
Egg viability %	r	0.245	0.957*	-0.009	-0.758	0.934	-	0.797	0.893
	P	0.755	0.043	0.991	0.242	0.066	-	0.203	0.107
Hatching %	r	-0.380	-0.710	-0.506	-0.592	0.669	0.797	-	0.957*
	P	0.620	0.290	0.494	0.408	0.331	0.203	-	0.043
Larval survival (3dph) %	r	-0.202	-0.874	-0.453	-0.796	0.849	0.893	0.957*	-
	P	0.798	0.126	0.547	0.204	0.151	0.107	0.043	-

The symbol *, ** denotes significant differences as $P < 0.05$ or 0.01 level

Table 5-20. Regression relationship analysis between sperm quality and egg quality of gilthead seabream broodstock (combined data from high (HG) or low growth (LG) groups fed with either FO or VO diet over three months of reproductive season

Regression analysis	Egg fertilization %		Egg viability %		Hatching %		Larval survival (3dph) %	
	R^2	P	R^2	P	R^2	P	R^2	P
Sperm concentration (10^9 cells/ml)	0.083	0.711	0.060	0.755	0.145	0.620	0.041	0.798
Sperm viability %	0.995	0.003	0.915	0.043	0.505	0.290	0.763	0.126
Sperm motility %	0.009	0.906	0.001	0.991	0.256	0.494	0.205	0.547
Sperm motility duration (sec)	0.863	0.071	0.574	0.242	0.350	0.408	0.633	0.204

5.3.2.4 Biochemical analysis

Egg proximate composition was affected by either broodstock selection or dietary fatty acid profile (Table 5-21). The highest protein and moisture contents and the lowest lipid contents were found in the eggs from LGVO broodstock, that were significantly different from those of LGFO broodstock (Table 5-21). The two-way ANOVA analysis showed the significant effect of the VO diet, increasing egg protein and moisture contents and reducing lipid contents (Table 5-22), being significantly different in the eggs from LG broodstock but not in those from HG broodstock, due to the interaction between Diet and Selection (Table 5-21).

Egg fatty acid composition markedly reflected the broodstock diet (Table 5-22). Thus, feeding the VO diet significantly reduced the egg contents in 16:1n-7, 16:2n-4, 16:4n-3, 18:1n-7, 18:1n-5, 18:2n-4, 18:3n-4, 18:4n-3, 18:4n-1, 20:1n-9, 20:1n-7, 20:1n-5, 20:3n-9, 20:4n-6, 20:3n-3, 20:4n-3, 20:5n-3, 22:1n-11, 22:4n-6, 22:5n-6, 22:5n-3, n-3/n-6 and 20:3n-6/20:2n-6, whereas it increased the egg contents in 18:1n-9, 18:2n-6, 18:3n-3, 20:2n-6, n-6 FA, n-9 FA, DHA/ARA and DHA/EPA (Table 5-22). However, certain fatty acids were contrary to their dietary levels such as 18:2n-9 and 20:2n-6 that were increased in the eggs from VO fed broodstock, and the ratios 20:2n-9/20:1n-9 and 20:4n-3/20:3n-3 that were respectively increased and reduced. Finally, despite the marked dietary changes in 22:6n-3, the level of this fatty acid was not significantly different in eggs of the different broodstock. Finally, the effect of selection on the eggs fatty acid profiles was very mild, and limited the fish fed FO diet (Table 5-22). For instance, in the eggs from HGFO broodstock the elongation product 20:2n-6 was increased in comparison to LGFO, whereas the desaturation ratios 16:4n-3, 18:4n-3, 18:4n-3/18:3n-3 were reduced (Table 5-22).

Table 5-21. Biochemical composition of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or VO diet over three months of reproductive season (HGFO, n=3; HGVO, n=3; LGFO, n=3; LGVO, n=3).

Egg biochemical composition	Broodfish groups				One-way ANOVA (*P* values)	Two-way ANOVA (*P* values)		
	HGFO	HGVO	LGFO	LGVO		Selection	Diet	S x D
Crude protein (%DM)	70.2 ± 0.4^{ab}	69.6 ± 2.6^{b}	69.3 ± 1.6^{b}	75.7 ± 1.1^{a}	0.016	0.062	0.044	0.023
Crude lipid (%DM)	23.8 ± 1.2^{a}	22.9 ± 0.7^{a}	23.8 ± 0.9^{a}	16.4 ± 0.8^{b}	<0.0001	0.003	0.001	0.002
Ash (%DM)	3.5 ± 0.3	3.6 ± 0.2	3.3 ± 0.2	3.6 ± 0.5	0.763	0.902	0.605	0.934
Moisture (%)	87.7 ± 0.3^{b}	87.7 ± 0.3^{b}	87.4 ± 0.1^{b}	89.2 ± 0.1^{a}	<0.0001	0.003	<0.001	0.001

Means bearing different superscript letters differ significantly (P<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 5-22. Fatty acid composition (% total fatty acids) of eggs of gilthead sea bream broodstock selected for high (HG) or low (LG) growth and fed with either FO or VO diet over three months of reproductive season

Fatty acid (%TFA)	HGFO	HGVO	LGFO	LGVO	One-way ANOVA (*P* value)	Two-way ANOVA (*P* value)		
						Selection	Diet	S x D
14:0	2.44±0.32	1.48±0.19	2.66±1.04	1.64±0.46	0.271	0.676	0.079	0.947
14:1n-7	0.02±0.00	0.01±0.00	0.02±0.01	0.03±0.03	0.606	0.507	0.820	0.292
14:1n-5	0.1±0.02	0.05±0.00	0.1±0.03	0.07±0.03	0.242	0.479	0.079	0.664
15:0	0.28±0.06	0.18±0.01	0.27±0.07	0.19±0.04	0.210	0.946	0.056	0.840
15:1n-5	0.01±0.00	0.02±0.01	0.02±0.01	0.02±0.01	0.734	0.460	0.460	1.000
16:0 ISO	0.06±0.01	0.03±0.00	0.05±0.01	0.04±0.01	0.136	0.756	0.040	0.374
16:0	16.79±3.61	13.01±0.71	15.28±1.42	13.53±0.04	0.337	0.741	0.119	0.507
16:1n-7	5.45±0.9^{ab}	3.09±0.31^{bc}	6.1±0.53^{a}	2.97±0.47^{c}	0.013	0.561	0.003	0.410
16:1n-5	0.11±0.01	0.07±0.00	0.1±0.04	0.07±0.01	0.306	0.733	0.094	0.733
16:2n-4	0.37±0.01^{a}	0.13±0.01^{b}	0.47±0.06^{a}	0.13±0.01^{b}	0.001	0.103	<0.001	0.131
17:0	0.28±0.01^{b}	0.12±0.00^{c}	0.36±0.02^{a}	0.1±0.00^{c}	<0.001	0.019	<0.001	0.003

16:3n-4	0.28±0.06	0.21±0.01	0.27±0.04	0.2±0.01	0.202	0.720	0.054	1.000
16:3n-3	0.13±0.02	0.07±0.01	0.11±0.01	0.07±±0.00	0.041	0.507	0.010	0.507
16:3n-1	0.1±0.03	0.07±0.01	0.08±0.01	0.06±0.02	0.229	0.249	0.102	0.595
16:4n-3	0.15±0.01^{b}	0.07±0.00^{c}	0.25±0.01^{a}	0.07±0.00^{c}	0.000	0.000	<0.001	0.000
18:0	5.44±1.2	3.93±0.09	4±0.45	4±0.04	0.189	0.206	0.170	0.172
18:1n-9	20.29±3.68^{ab}	28.09±1.62^{a}	18.26±0.95^{b}	28.73±1.73^{ab}	0.020	0.685	0.004	0.446
18:1n-7	3.58±0.64	2.57±0.01	3.81±0.35	2.71±0.23	0.073	0.534	0.018	0.863
18:1n-5	0.21±0.04	0.13±0.00	0.18±0.03	0.14±0.01	0.077	0.570	0.021	0.407
18:2n-9	0.14±0.01	0.2±0.03	0.14±0.02	0.19±0.01	0.065	0.506	0.016	0.733
18.2n-6	6.69±0.74^{b}	11.81±0.65^{a}	5.79±0.2^{b}	12.11±0.74^{a}	0.001	0.537	<0.001	0.247
18:2n-4	0.27±0.04^{a}	0.1±0.01^{b}	0.28±0.02^{a}	0.08±±0.00^{b}	0.001	0.874	<0.001	0.446
18:3n-6	0.22±0.04	0.25±0.04	0.23±0.01	0.24±0.00	0.636	1.000	0.273	0.638
18:3n-4	0.26±0.03^{a}	0.13±0.02^{b}	0.26±0.04^{a}	0.12±0.00^{b}	0.008	0.791	0.002	1.000
18:3n-3	1.51±0.01^{a}	4.96±0.76^{b}	1.44±0.02^{a}	5.58±0.79^{b}	0.003	0.522	0.001	0.422
18:4n-3	0.87±0.16^{ab}	0.53±0.07^{b}	1.21±0.06^{a}	0.56±0.06^{b}	0.006	0.054	0.002	0.080
18:4n-1	0.2±0.04^{a}	0.08±0.01^{b}	0.25±±0.00^{a}	0.07±0.01^{b}	0.003	0.233	0.001	0.161
20:0	0.14±0.04	0.07±0.00	0.06±0.08	0.08±0.00	0.432	0.356	0.497	0.251
20:1n-9	0.24±0.04^{a}	0.13±0.01^{b}	0.22±0.01^{a}	0.13±0.01^{b}	0.012	0.468	0.003	0.468
20:1n-7	1.11±0.18	0.86±0.04	0.97±0.06	0.87±0.02	0.158	0.405	0.058	0.341
20:1n-5	0.2±0.03^{a}	0.1±0.00^{b}	0.18±0.00^{a}	0.1±0.00^{b}	0.004	0.374	0.001	0.374
20:2n-6	0.27±0.04^{ab}	0.34±0.02^{a}	0.21±0.01^{b}	0.32±0.01^{a}	0.013	0.074	0.004	0.223
20:3n-9	0.05±0.01^{ab}	0.03±0.01^{b}	0.06±0.00^{a}	0.03±0.00^{b}	0.007	0.047	0.002	0.230
20:3n-6	0.18±0.00	0.17±0.01	0.17±0.01	0.17±0.00	0.702	0.519	0.519	0.519
20:4n-6	1.04±0.11^{a}	0.6±0.04^{b}	0.94±0.01^{a}	0.54±0.02^{b}	0.002	0.116	0.001	0.693
20:3n-3	0.20±0.01^{b}	0.33±0.01^{a}	0.17±0.01^{b}	0.34±0.01^{a}	<0.001	0.452	<0.001	0.124
20:4n-3	1.00±0.15^{a}	0.65±0.04^{b}	1.13±0.06^{a}	0.65±0.01^{b}	0.008	0.306	0.002	0.306
20:5n-3	8.52±2.55	5.14±0.46	10.3±1.24	5.07±0.25	0.051	0.449	0.013	0.415
22:1n-11	0.35±0.04^{a}	0.17±0b	0.32±0.04^{a}	0.17±0.01^{b}	0.006	0.434	0.001	0.434

22:1n-9	0.13±0.01	0.11±0.03	0.11±0.01	0.13±0.05	0.869	0.912	0.912	0.459
22:4n-6	0.1±0.01^{ab}	0.06±0.02^{ab}	0.11±0.01^{a}	0.05±0.01^{b}	0.025	0.809	0.006	0.482
22:5n-6	0.27±0.06^{a}	0.16±0.03^{b}	0.28±0.02^{a}	0.16±0.02^{b}	0.043	1.000	0.010	0.850
22:5n-3	2.72±0.99	1.95±0.3	3.21±0.37	1.74±0.31	0.170	0.747	0.050	0.442
22:6n-3	17.24±7.69	17.76±3.29	19.58±3.12	15.75±3.83	0.884	0.965	0.655	0.561
Total Saturates	25.35±5.23	18.78±0.83	22.62±3.09	19.52±0.42	0.266	0.671	0.090	0.468
Total Monoenes	31.87±5.58	35.44±1.85	30.48±1.36	36.16±2.39	0.357	0.892	0.114	0.669
Total n-3	32.32±11.51	31.44±3.28	37.39±4.78	29.81±3.51	0.714	0.734	0.421	0.517
Total n-6	8.66±0.64^{b}	13.33±0.59^{a}	7.61±0.21^{b}	13.53±0.68^{a}	0.001	0.343	<0.001	0.187
Total n-9	20.91±3.75^{ab}	28.62±1.61^{a}	18.84±1^{b}	29.26±1.65^{a}	0.021	0.677	0.005	0.442
Total n-3 LC-PUFA	29.67±11.38	25.81±4.1	34.39±4.77	23.54±4.36	0.493	0.814	0.204	0.511
EPA+DHA	25.76±10.23	22.89±3.75	29.88±4.36	20.81±4.07	0.556	0.828	0.246	0.519
ARA/EPA	0.13±0.02	0.12±0.00	0.09±0.01	0.11±0.01	0.168	0.056	0.621	0.345
EPA/ARA	8.15±1.63	8.56±0.16	11.01±1.25	9.47±0.09	0.157	0.061	0.480	0.253
DHA/ARA	16.37±5.77	29.47±3.39	20.91±3.2	29.32±5.99	0.120	0.551	0.033	0.525
DHA/EPA	1.98±0.31^{ab}	3.45±0.33^{a}	1.9±0.08^{b}	3.1±0.6^{ab}	0.033	0.463	0.008	0.647
n-3/n-6	3.79±1.61	2.37±0.35	4.93±0.76	2.21±0.37	0.114	0.497	0.034	0.378
18:4n-3/18:3n-3	0.58±0.11^{b}	0.11±0.00^{c}	0.85±0.05^{a}	0.1±0.00^{c}	0.001	0.043	<0.001	0.035
20:2n-9/20:1n-9	0.31±0.07^{b}	0.56±0.03^{a}	0.29±0.01^{b}	0.52±0.03^{a}	0.005	0.323	0.001	0.808
20:3n-6/20:2n-6	0.69±0.1^{ab}	0.51±0.01^{b}	0.82±0.02^{a}	0.54±0.02^{b}	0.012	0.102	0.003	0.265
20:4n-3/20:3n-3	5.03±0.49^{a}	1.96±0.02^{b}	6.62±0.98^{a}	1.9±0.06^{b}	0.002	0.119	0.001	0.101

5.4 Discussion

Genetic selection programmes with teleosts had focussed much on the improvement of growth rates, but in recent years, many other productive traits have been used (Gjedrem, 2012; Gjedrem and Rye, 2018). The major traits used in selective breeding in fish include feed efficiency, skeletal deformities, disease resistance, fillet yield, and flesh and carcass quality (Besson et al., 2019; Chavanne et al., 2016; De Verdal et al., 2018; García-Celdrán et al., 2016; Janssen et al., 2017; Navarro et al., 2009). In genomic selection, DNA maker-based information is used to predict the breeding value of different genotyped traits (Meuwissen et al., 2013). This approach has shown accurate prediction of breeding value for the growth trait as compared to conventional methods of selection and has been widely adopted in salmonids (Bangera et al., 2017; Yoshida et al., 2018). In recent times, the improvement of feed efficiency through genetic selection programs has also gained much attention (Besson et al., 2019; Callet et al., 2017; De Verdal et al., 2018). Similarly, efforts towards selection of fish for better utilisation of plant-based diets have also been made (Le Boucher et al., 2013, 2012, 2011; Perera et al., 2019). It is also reported that feeding diets containing very low levels of fish meal and fish oil over the full life cycle, from early life to broodstock, in the gilthead seabream does not affect growth (Simó-Mirabet et al., 2018). The latter authors unfortunately did not look into the reproductive performance of gilthead seabream fed such diets. A high nutritional plasticity has been reported in this protandrous hermaphroditic fish (Menoyo et al., 2004; Monge-Ortiz et al., 2016; Perera et al., 2019). The above-mentioned studies with gilthead seabream focused on the growth traits and other physiological and metabolic parameters, without addressing whether broodstock selection for growth trait and the utilisation of fish oil or vegetable oil based diets affects reproductive performance.

Marine teleosts show limited ability to bio-convert LC-PUFAs from LA and ALA substrate due to low expression of the *fads2* gene with a low activity of Fads enzyme (Monroig et al., 2011; Tocher, 2015). This attribute can significantly affect the reproductive performance in fish, if sufficient amount of essential fatty acids is not supplied in the broodstock diet. There are few strategies applied to improve the spawning quality in fish under low fish meal and fish oil feeding regimes through broodstock nutritional programming (Ferosekhan et al., 2020; Izquierdo et al., 2015; Lazzarotto et al., 2016; Turkmen et al., 2020; Xu et al., 2019). We conducted the mass spawning of gilthead seabream broodstock of either high or low growth broodstock under two feeding regimes (FO or VO diet). The body weight of HG broodstock was significantly higher than that of the LG broodstock, well in conformity with the selection for growth trait, and we maintained an equal broodstock biomass for both HG and LG groups.

Since the broodstock diet is known to have a strong influence on spawning quality in fish, the formulated broodstock diet in our experiment contained same level of ARA, EPA and DHA in the FO and VO diets used for gilthead seabream broodstock as in earlier studies (Ferosekhan et al., 2020; Izquierdo et al., 2015; Turkmen et al., 2020; Xu et al., 2019). The broodstock selected for high growth showed higher GSI, supporting the production of higher number of eggs by the HG broodstock under commercial diet feeding regime. Likewise, HSI was found to be higher for female broodstock in this study, which may suggest the higher requirement of vitellogenin synthesis in the female liver for the production of lipoproteins, particularly phosphovitine and lipovitellin rich in n-3 LC-PUFA, which transport lipids to the developing oocyte. These results agree with the higher GSI observed in female in comparison to males as reported earlier (Chaoui et al., 2006; Kissil et al., 2001; Zohar et al., 1978). Testosterone, a major hormone which regulates the spermatogenesis process from spermatogonial proliferation to spermatocyte formation in fish through endocrine pathway (Billard et al., 1974), was significantly higher in male HG broodstock. Plasma steroid hormone levels of both male and female gilthead seabream showed values as reported in other studies (Chaves-Pozo et al., 2008; Meiri et al., 2002; Mosconi et al., 2002).

11-ketotestosterone is the main androgen, controlling spermatogenesis and also secondary sexual characteristics in males (Borg, 1994; Hishida and Kawamoto, 1970). 11KT is synthesised from testosterone and our data show a strong positive correlation between plasma testosterone and 11KT levels in male seabream broodstock. Also, in the females, we found plasma testosterone and 17β-estradiol levels had a slight correlation, as 17β-estradiol production is dependent on testosterone as a substrate (Nagahama, 2002; Ozon, 1972; Yaron and Levavi-Sivan, 2011). The broodstock size had no relation to the plasma steroid levels in gilthead seabream broodstock as reported earlier in rainbow trout (Scott et al., 1980) or Atlantic salmon (Dodd et al., 1978). The HG broodstock always had a relatively high spawning quality. This is in agreement with data from other studies where it is observed that bigger size broodstock produces higher number of eggs and larvae in fish as reported in seabream (Jerez et al., 2012), channel catfish (Davis et al., 2005), rainbow trout (Pitman, 1979), Atlantic salmon (Rollinson and Hutchings, 2010), African catfish (Sule and Adikwu, 2004).

The fatty acid composition of broodstock diet is known to play a significant role in determining egg and larval quality in fish (Izquierdo et al., 2015, 2001). We observed few spawning quality parameters to be of similar magnitude in both HG or LG broodstock. This might be due to the feeding with similar fatty acid composition diet in the commercial diet study period. As we know, the same diet composition will always produce equal quality of

eggs and egg composition unless when particular strategies are applied such as broodstock selection for *fads2*, which can improve the reproductive performance in seabream even when fed a VO diet (Ferosekhan et al., 2020; Izquierdo et al., 2015; Turkmen et al., 2020; Xu et al., 2019). Female steroid hormone 17β-estradiol level was significantly improved by the experimental diet as compared to commercial diet. A high dietary supply of fatty acid precursors increases the conversion of ALA to n-3 LC-PUFA promoted by oestrogen in pregnant women, claimed to be important to fulfil the essential fatty acid requirements of foetus and neonate (Burdge and Calder, 2005).

Although less well studied, it is known since long that that sperm quality is also a very important variable in broodstock management in fish, with a strong influence on egg fertilisation rate (Billard et al., 1974; Bromage, 1995; Cabrita et al., 2005). In general, the sperm quality increases with age and size of fish and reported maximum quality to certain sizes and further increase in age or size leads to reduction in sperm quality (And and Zohar, 1999; Liley et al., 2002; Risopatrón et al., 2018). In rainbow trout, it is shown that two to three year old broodstock has good sperm quality and beyond this age, the sperm quality is drastically decreased (Risopatrón et al., 2018). We also found that sperm cell concentration of seabream broodstock was significantly higher in the higher growth/bigger sized than in the lower growth/smaller sized broodstock. In some terrestrial animals, sperm cell concentration is reported to have a positive correlation to sperm motility (Love et al., 2003) as observed in our study. The sperm viability is directly influenced by sperm motility duration, which helps the sperm to search for the eggs to enter through egg micropyle and fuses with the oocyte plasma membrane to fertilize the eggs (Billard et al., 1974; Ginsburg, 1963; Kobayashi and Yamamoto, 1981). Our data showed that in seabream broodstock, sperm viability significantly increased egg fertilisation and viability rate generally observed in fish (Ciereszko and Dabrowski, 1994; Moccia and Munkittrick, 1987), although such a relation was not observed in some species such as the Atlantic salmon (Aas et al., 1991), sockeye salmon (Hoysak and Liley, 2001), or Atlantic cod (Trippel and Neilson, 1992). The dietary fatty acid composition influences the sperm quality in fish (Beirão et al., 2015; Bobe and Labbé, 2010; Labbé et al., 1995, 1991; Rurangwa et al., 2004). In this study, we observed that sperm quality is not much affected by the parental diet fatty acid profile. This may be that the level of essential fatty acids (EFAs) present in both FO and VO diets was sufficient enough for normal spermatogenesis in gilthead seabream (Ferosekhan et al., 2020). The dietary ARA, EPA and DHA levels used here were found to be more or less at the same level than in studies with other fish species such as European sea bass (Asturiano et al., 2001), Senegalese sole (Beirão et al., 2015), rainbow trout

(Köprücü et al., 2015), European eel (Butts et al., 2015), Siberian sturgeon (Luo et al., 2017) or Eurasian perch (Henrotte et al., 2010).

The continuous spawners like gilthead seabream have very short vitellogenic periods and the spawning quality is directly affected by the parental dietary fatty acid composition (Fernández-Palacios et al., 1995; Izquierdo et al., 2015; Zohar et al., 1978). It has been suggested that the biochemical composition of fish eggs is related to the spawning quality as egg reserves must satisfy embryonic development (Harel et al., 1994; Izquierdo et al., 2001). Gilthead seabream females continue to actively feed during sexual maturation throughout the spawning season and produce an egg biomass greater than their own body weight, which require greater amount of EFAs during the spawning period (Fernández-Palacios et al., 1995; Izquierdo et al., 2015, 2001). DHA, as an EFA, plays a more important role in the enzyme activity of the cell membrane and in physiological balance than EPA. Deficiencies in DHA could lead to reduction in egg and larval quality (Bell et al., 1997; Fernández-Palacios et al., 1995; Izquierdo et al., 2015, 2001). Study confirmed for gilthead seabream that larvae had preferentially conserved DHA over EPA during deprivation, which indicates essentiality of DHA in the parental diet (Koven et al., 1998). In our study also, we observed that FO diet had higher DHA and EPA than VO diet. In turn, the higher DHA and EPA of parental FO diet significantly improved the spawning quality in seabream broodstock as reported in the above studies.

Moreover, DHA content in the eggs was effectively increased in females fed with FO diet and also broodstock selected for high growth had relatively higher amount of DHA than LG broodstock. It indicates an efficient bioconversion or specific retention of fatty acids from diet to eggs as seen in many fish. The reduced DHA, EPA and ARA levels in the VO diet led to a significant reduction in number of egg and larval production in gilthead seabream. In many species, egg viability is an ideal indicator to ascertain the egg quality. It was observed that broodstock selected for high growth and fed with FO diet (HGFO, 81%) had produced 27% more viable eggs as compared to low growth broodstock fed with VO diet (LGVO, 54%). This is a strong evidence that indicate that a low level of EFAs in parental diet reduces the egg and larval viability (Izquierdo et al., 2015). The higher DHA/EPA ratios in the eggs also suggest the activation of the Sprecher pathway to synthesise DHA after beta-oxidation from 24:6n-3 (Oboh et al., 2017). Increase in egg DHA content by FO diet in females would lead to an increased production of docosanoids, which also play an important role in induction of oocyte maturation, improving fecundity in terms of eggs produced (Ann Sorbera et al., 2001). FO replacement by combination of linseed and rapeseed oil in parental diet reduced the amount of

EFAs and increase in 18:2n-6 and 18:3n-3 precursors, which tailor the gilthead seabream offspring to produce juveniles to better utilize the low FM and FO diets (Turkmen et al., 2020). Additionally, it was noticed that gilthead seabream broodstock selected for high growth certainly has an improved spawning quality particularly when the broodstock is fed with sufficient levels of essential fatty acids.

5.5 Conclusion

This study emphasizes the strong positive effect of dietary fatty acids on the reproductive performance, egg and larval quality of gilthead seabream broodstock. The high growth (HG) trait gilthead seabream broodstock was found to produce higher number of sperm cells and had increased sperm motility. This group had significantly higher egg viability percentage, which ultimately produced relatively higher number of eggs and larvae. The steroid hormone production, sperm and egg quality was markedly improved in the broodstock selected for high growth and fed with fish oil-based diet. The egg viability and number of eggs and larvae production was also significantly improved by the dietary fatty acid of FO diet and to some extent by broodstock selection. This study clearly indicates that gilthead seabream broodstock selected on growth trait could have positive role in improvement of sperm and egg quality to produce viable progeny.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 (Blue Growth) H2020-BG-2018-818367 (AquaIMPACT project) and PROGENSA III (Mejora de la competitividad del sector de la dorada a través de la selección genética, JACUMAR program with support from the European Maritime and Fisheries). The first author gratefully acknowledges the financial assistance provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India for the doctoral study. This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein.

Authors' contribution

Conceived and designed the experiment: MI SF SK. Broodstock selection: MI SF JMA CPG. Diet formulation and preparation: MI SF RF SK. Steroid hormone analysis: SF AGZ. Sperm quality evaluation: SF NS AGA ST. Egg and larval quality evaluation: SF CPG NS. Biochemical analysis SF ST HX. Data analysis: SF MI SK. Wrote the paper: SF MI SK.

CHAPTER 6

Selection for high growth improves reproductive performance of gilthead seabream *Sparus aurata* under mass spawning conditions, regardless of the dietary lipid source

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Submitted to *Animal Reproduction Science* Journal (IF: 2.145)

Chapter 6

Abstract

Genetic selection programmes in gilthead seabream mainly focus on traits related to growth, disease resistance, skeletal anomalies or fillet quality. However, the effect of selection for growth on the reproductive performance of seabream broodstock has not received much attention. The present study aimed to determine the effect of selection for growth traits, high (HG) or low (LG) growth, and broodstock feeding with fish oil (FO diet) or rapeseed oil (RO diet) as main lipid sources, on reproductive performance of gilthead seabream. For the first part of the spawning season (Phase I) HG and LG broodstock were fed a commercial diet and the HG broodstock produced a higher number of larvae and higher viable eggs, hatching and larval survival rates than LG broodstock, affecting egg fatty acid profiles. For the second part of the study (Phase II) broodstock were fed one of the two diets containing FO or RO. Fecundity in terms of viable eggs, hatchlings, and larvae produced, as well as fertilization rates, were improved in HG broodstock. Some fatty acids such as 18:0, 20:2n-6, 20:3n-3 or EPA/ARA were also affected by the growth selection. According to the two-way ANOVA analysis, feeding the RO diet did not significantly affect fecundity parameters, but slightly reduced fertilization and hatching rates in HG broodstock. Nevertheless, HG broodstock showed better spawning quality parameters than LG broodstock, even when they were fed the RO diet. Egg fatty acid profiles reflected diet composition, although DHA contents were not affected. In conclusion, broodstock selected for high growth had a positive effect on broodstock performance, and FO replacement by RO did not markedly affect reproduction providing that fatty acid contents were sufficient to fulfil the essential fatty acid requirements of gilthead seabream broodstock.

Keywords: Broodstock, fatty acids, fish oil replacement, rapeseed oil, genetic selection, egg and larval quality

6.1 Introduction

Gilthead seabream (*Sparus aurata*) is the major farmed marine fish in the European Union and Mediterranean region (FAO, 2020). Genetic improvement either through mass selection or family selection is practiced in different species of fish in order to improve growth (Fernandes et al., 2017; Regan et al., 2021; Gjedrem, 2012), disease resistance (Palaiokostas et al., 2016; Fjalestad et al., 1993), fillet quality and pigmentation (Horn et al., 2020; Garcia et al., 2017; Schlicht et al., 2019) or to reduce skeletal anomalies (Negrín-Báez et al., 2015; García-Celdrán et al., 2016; Lorenzo-Felipe et al., 2021). In gilthead seabream, genetic selection programmes have tried to address several traits of importance such as improved growth (Borrell et al., 2011), disease resistance (Piazzon et al., 2020) or reduced skeletal anomalies (García-Celdrán et al., 2016; Lorenzo-Felipe et al., 2021). Most of the selection programmes in fish are based on mass selection due to easy implication and management (Gorshkov et al., 1997). The genetic improvement programme in gilthead seabream has led to a 5-29% increase in growth rate per generation, which eventually produced the genetically improved seeds for better performance in aquaculture conditions (Knibb et al., 1997; Brown et al., 2005). It was estimated that around 40% of seeds used in the European aquaculture are mostly coming from selective breeding programmes (Chavanne et al., 2016). PROGENSA, a Spanish National Breeding Program (Afonso et al., 2012; García-Celdrán et al., 2015; Lee-Montero et al., 2015; Lorenzo-Felipe et al., 2021), is an ongoing selective breeding programme intended to improve the growth and skeletal anomalies of seabream through the Best Linear Unbiased Prediction (BLUP) methodology (Lorenzo-Felipe et al., 2021).

Gilthead seabream is a batch spawner whose egg quality is affected by the broodstock diet composition (Fernández-Palacios et al., 2011). Besides, broodstock nutrition plays an important role in gamete quality and offspring performance (Izquierdo et al., 2001; Fernández-Palacios et al., 1995). Among other nutrients, lipids and fatty acid (FA) contents of broodstock diets have been identified as major dietary contributors for ensuring successful reproduction and the offspring survival (Izquierdo et al., 2001). The importance of broodstock diet composition in the development of the testis, ovary, oocyte quality and offspring performance is widely studied and reported for many fish species, particularly in improving the reproductive performance of gilthead seabream (Fernández-Palacios et al., 1995; Izquierdo et al., 2015; Xu et al., 2019; Ferosekhan et al., 2020, 2021). The fatty acid composition of fish eggs reflects that of the parental diet and long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic

acid (ARA; 20:4n-6), are required in broodstock diets to improve the egg and larval quality (Fernández-Palacios et al., 1995, Izquierdo et al., 2001). Thus, during gametogenesis, LC-PUFAs are preferentially incorporated into ova (Bromage, 1995; Harel et al., 1994). However, marine fish have a limited ability to synthetize these essential fatty acids (EFA), from their fatty acid precursors linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) (Monroig et al., 2011; Oboh et al., 2017; Tocher, 2015). Therefore, it is considered necessary to include sufficient amounts of these EFA for improving growth, health, immunity, reproduction, gamete quality and offspring performance of fish (Izquierdo and Koven, 2011; Tocher, 2015). Dietary supply of EPA and DHA are also essential to support neural and visual development, besides many physiological and behavioural activities (Benitez-Santana et al., 2014). Fish oils (FO) were traditionally being used as the main sources of dietary n-3 LC-PUFA, but their production has become stagnant and their price and demand have increased, and therefore at present, different alternative oils are replacing FO in fish diets. However, most of these oils are plant oils devoid of the essential LC-PUFAs, although they contain the ALA and LA precursors. Therefore, several strategies are being developed to improve the LC-PUFA synthesis from those precursors (Izquierdo et al., 2015). Nutritional programming of the offspring, through the modification of the broodstock diet, effectively stimulates LC-PUFA synthesis and improves the use of low fish meal (FM) and low FO diets along on-growing (Izquierdo et al., 2015; Turkmen et al., 2020). Besides, offspring from broodstock selected for high expression of fatty acyl desaturase 2 gene (*fads2*) also exhibit a better ability for improved utilization of low FM/FO diet (Xu et al., 2019, 2021; Turkmen et al., 2019). Moreover, the nutritional programing effect persisted even in the 16-months old gilthead seabream offspring, which could utilize a very low FM/FO diet maintaining a good growth and an adequate fillet fatty acid profile (Turkmen et al., 2017b). Recently, gilthead seabream broodfish were fed a low FO diet to nutritionally program the offspring to improve the use of low FO diets along on-growing (Ferosekhan et al., 2021). In such nutritional programing diet, FO was completely replaced by 1.5% linseed oil (LO) and 8.5% rapeseed oil (RO), oils rich in ALA (9.58% total fatty acids, TFA) and LA (13.49% TFA), both fatty acids precursors of LC-PUFA, to stimulate LC-PUFA biosynthesis. However, feeding this diet significantly reduced all fecundity and spawning quality parameters, regardless the selection for high (HG) or low (LG) growth (Ferosekhan et al., 2021). Since, the dietary levels of EPA, DHA and ARA were sufficient to fulfil the EFA requirements of gilthead seabream broodstock, the poor reproductive performance was most probably linked to an excessive dietary content of linoleic and, particularly, linolenic acid.

Based on these findings, the present study was conducted to evaluate the reproductive performance of gilthead seabream selected for high growth and fed an improved diet under mass spawning conditions. Therefore, the dietary content of LA and ALA was reduced by removing linseed oil, decreasing rapeseed oil to 7.54% and adding 1.76% fish oil, to reduce the possible negative effects of a diet totally devoid of FO on broodstock performance. This approach has direct implications for the gilthead seabream hatcheries for improved reproductive and offspring quality through appropriate broodstock selection programmes. In this context, the present study was carried out to delineate the effect of dietary fatty acid profiles on spawning quality, larval performance, biochemical and fatty acid composition of the eggs from broodstock of gilthead seabream selected for improved growth and reared under mass spawning conditions.

6.2 Materials and methods

6.2.1 Ethical statement

The study was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at Aquaculture Research Group (GIA) of ECOAQUA Institute, University of Las Palmas de Gran Canaria (ULPGC), Canary Islands, Spain. All experimentation performed at the (ULPGC) was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: OEBA-ULPGC-20/2018 R1).

6.2.2 Broodstock selection and management

The gilthead seabream broodstock used originated from the third generation of selection under the PROGENSA (Spanish National Breeding Program) project (Afonso et al., 2012; García-Celdrán et al., 2015; Lee-Montero et al., 2015; Lorenzo-Felipe et al., 2021). Two broodstock groups expressing either high growth (H) or low growth (L), selected by Best Linear Unbiased Prediction (BLUP) methodology with VCE-v 6.0 software (Neumaier and Groeneveld, 1998), were used for the assessment of reproductive performance under mass spawning conditions. High and low growth trait broodstock fish were individually marked with PIT tags (EID Iberica SA-TROVAN, Madrid, Spain) and maintained separately for mass spawning in four tanks (10 m^3) at the facilities of ECOAQUA institute (ULPGC, Canary Islands, Spain). All the tanks were supplied with seawater (37 g L^{-1} salinity, 17.0-19.0 °C) with

a daily water exchange of 600% and maintained under natural photoperiod. The four broodstock groups were maintained separately for the whole reproductive season as HG (tanks 1 and 2) and LG (tanks 3 and 4), with average biomass of 58 kg/tank and an average ratio of female/male biomass range from 1.5 to 2.2 (Table 6-1). Male and female body weight and length were larger in H than L broodstock (Table 6-1).

Table 6-1. Description of gilthead seabream broodstock selected for mass spawning experiment.

Broodstock details	HGFO	HGRO	LGFO	LGRO
Broodstock density/tank				
Male (n)	30	30	28	28
Female (n)	14	18	16	17
Total (n)	44	48	44	45
Broodstock biometry				
Male length (cm)	43.05±1.69	42.71±1.88	42.20±3.42	41.18±2.95
Male weight (kg)	1.34±0.21	1.35±0.17	1.24±0.28	1.15±0.26
Female length (cm)	43.26±2.14	43.43±2.78	39.56±2.12	41.19±2.40
Female weight (kg)	1.38±0.27	1.49±0.28	1.03±0.16	1.15±0.23
Male biomass (kg)	40	41	35	32
Female biomass (kg)	20	27	16	20
Total biomass (kg)	60	67	51	52
Female / male biomass (kg)	2.0	1.5	2.1	1.6

6.2.3 Phase I: Evaluation of mass spawning quality before feeding the experimental diet

The selected male and female broodfish from HG and LG groups were assessed for spawning quality. At the beginning of the spawning season from 30 December 2019 to 22 January 2020 all the broodfish groups were fed with a commercial diet (Crude protein 57.1%, Crude lipid 20.8%, and Ash 9.3%; Skretting, Burgos, Spain) to ensure that there were no significant differences in the spawning quality among broodfish from the same selection group (HG or LG). The fatty acid composition of the commercial diet is provided in table 6-3. For the evaluation of spawning quality, the spontaneously spawned eggs from each broodstock, HG or LG group were collected six times per week and estimated for all the spawning quality parameters as described in Ferosekhan et al. (2020, 2021). Fertilized eggs were collected at the end of the feeding period and kept at -80ºC for chemical and fatty acid composition analyses.

6.2.4 Phase II: Evaluation of mass spawning quality after feeding the experimental diets

The experimental broodstock feeds were formulated to be iso-proteic and iso-lipidic with either fish oil (FO diet) or a mixture of fish and rapeseed oils (RO diet) as the lipid source and were produced by Skretting ARC, Stavanger, Norway (Tables 6-2 and 6-3). Compared to the FO diet, the RO diet had higher levels of 18:2n-6 and 18:3n-3 fatty acids and reduced levels of saturated, monoenoic, and n−3 LC-PUFA (20:5n−3; eicosapentaenoic acid, EPA and 22:6n−3; docosahexaenoic acid, DHA) (Table 6-3). The broodstock treatment groups were assigned as follows: HGFO, HGRO, LGFO, and LGRO. Both the HG and LG broodfish groups were fed with one of the two diets at the rate of 1% body weight, twice a day (9:00 and 14:00 h), over 3 months (24 February 2020 to 16 April 2020). Seawater temperature during the spawning period was in the range of 18.5-23 °C and fish were kept under natural photoperiod. Egg collection for spawning quality and biochemical composition followed the same protocol described in the commercial diet feeding phase (Ferosekhan et al., 2020, 2021). Finally, after 3 months of feeding the two diets, eggs were collected from all broodfish groups (HGFO, HGRO, LGFO, and LGRO) and analysed for egg biochemical and fatty acid composition.

Table 6-2. Ingredients and proximate composition of the broodstock experimental diets used for mass spawning study.

Feed ingredients (%)	Diet FO	Diet RO
Fish meal (North-Atlantic 12C)	59.36	59.36
Krill meal	7.00	7.00
Squid meal	3.00	3.00
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 (% dry matter, DM)	58.9	58.1
Crude lipid (% DM)	21.3	22.1
Ash (% DM)	9.8	9.8

Table 6-3. Fatty acid profiles (% total fatty acids) of commercial, FO and RO broodstock diets.

%TFA	Commercial diet	FO diet	RO diet
14:0	2.74	6.01	2.22
14:1n-7	0.02	0.03	0.01

14:1n-5	0.11	0.20	0.07
15:0	0.30	0.45	0.18
15:1n-5	0.02	0.03	0.01
16:0 ISO	0.05	0.08	0.03
16:0	12.70	17.65	9.42
16:1n-7	3.74	6.93	2.20
16:1n-5	0.12	0.21	0.07
16:2n-6	0.01	0.02	0.01
16:2n-4	0.30	0.85	0.24
17:0	0.26	0.99	0.23
16:3n-4	0.21	0.14	0.10
16:3n-3	0.12	0.16	0.07
16:3n-1	0.03	0.07	0.03
16:4n-3	0.36	1.08	0.33
16:4n-1	0.01	0.01	0.01
18:0	3.13	3.45	2.41
18:1n-9	27.58	9.96	36.94
18:1n-7	2.67	2.70	2.74
18.1n-5	0.15	0.20	0.14
18:2n-9	0.06	0.10	0.02
18.2n-6	12.08	2.64	12.32
18:2n-4	0.14	0.26	0.08
18:3n-6	0.15	0.24	0.08
18:3n-4	0.17	0.26	0.10
18:3n-3	3.97	1.12	4.85
18.3n-1	0.02	0.05	0.02
18:4n-3	1.15	2.70	1.30
18:4n-1	0.12	0.20	0.07
20:0	0.37	0.29	0.48
20:1n-9	0.30	0.30	0.23
20:1n-7	3.34	3.70	3.81
20:1n-5	0.19	0.27	0.12
20:2n-9	0.10	0.12	0.02
20:2n-6	0.68	0.20	0.14
20:3n-9	0.03	0.07	0.03
20:3n-6	0.16	0.13	0.04
20:4n-6	0.58	0.94	0.35
20:3n-3	0.34	0.13	0.08
20:4n-3	0.71	0.82	0.29
20:5n-3	6.03	13.47	5.26
22:1n-11	2.96	4.37	3.92
22:1n-9	0.55	0.43	0.78
22:4n-6	0.11	0.13	0.05
22:5n-6	0.24	0.32	0.13
22:5n-3	1.33	1.83	0.52

22:6n-3	9.49	13.71	7.46
Total saturates	19.49	28.84	14.94
Total monoenes	41.76	29.32	51.04
Total n-3	23.50	35.02	20.16
Total n-6	14.01	4.61	13.11
Total n-9	28.59	10.90	37.99
Sum n-3 LC-PUFA	17.90	29.96	13.61
EPA/ARA	10.43	14.39	15.08
DHA/EPA	1.57	1.02	1.42
DHA/ARA	16.41	14.65	21.39
n-3/n-6	1.68	7.60	1.54

6.2.5 Evaluation of egg and larval quality

The naturally spawned eggs after 20 hours of incubation were collected and placed in a 10 L container provided with aeration. Egg samples (n=3; 10 mL) were randomly collected with thorough mixing and placed in a Bogorov chamber under the light microscope to calculate the total number of eggs and percentages of fertilized and viable eggs. Egg viability was determined by observing the percentage of morphologically normal eggs after 1-day post-fertilization (1 dpf) (Fernández-Palacios et al., 2011). Then, the viable eggs were individually placed in 96-well microtiter plates in two replicates filled with filtered and UV sterilized seawater. Eggs were incubated in a controlled temperature incubator at 19-21 °C to estimate the percentage of hatching (2 dpf) and larval survival rates at 3 days post-hatch (dph). From these values, other fecundity parameters were calculated, including total numbers of fertilized, viable, hatched, and larvae produced per kg female (Fernández-Palacios et al., 1995, Ferosekhan et al., 2020, 2021).

6.2.6 Egg biochemical analysis

After feeding either the commercial diet or the experimental diets, egg samples were collected from all the broodstock groups and stored at -80°C for analysis of proximate and fatty acid composition. Moisture contents were obtained after drying the samples in an oven at 110°C for 24 h and then for 1 h until constant weight. Ash content was determined after incineration at 600°C for 16 h. Crude protein content was determined by measuring the N content (N×6·25) through automated Kjeldahl analysis (AOAC, 1995) and crude lipid extraction was carried out with chloroform: methanol (Folch et al., 1957). Fatty acid methyl esters (FAMEs) from egg total lipids were prepared by transmethylation method with 1%

sulfuric acid in methanol, purified on NH2 silica (Sep-pak; Waters), and separated and quantified in a gas chromatograph (GC14A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Carbowax 20 M (30 m x 0.32 mm x 0.27 m) silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column initial temperature was set to 170 ºC for 10 min and then it was raised to 220 ºC at 2.5 ºC per min and finally maintained at 215 ºC for a further 5 min. FAMEs were identified by comparison with previously characterized standards. Specific unclear peaks were identified by GLC-MS (TRACETM GC Ultra and PolarisQ mass spectrometer; Thermo Fisher Scientific) (Izquierdo et al., 1989).

6.2.7 Statistical analysis

Data are reported as mean ± standard deviation (SD). Data were compared statistically using the analysis of variance (ANOVA), at a significance level of < 0.05. All variables were checked for normality and homogeneity of variance (Sokal and Rohlf, 1979). Data which did not fit for normality was subjected to an arcsin transformation to attain normality. When arcsin-transformed data were not normally distributed, then Kruskal–Wallis, a non-parametric test was applied to the non-transformed data. An independent sample *t*-test was performed to compare, spawning quality, egg biochemical, and fatty acid composition for the commercial diet feeding phase to check the broodstock selection (HG or LG) effect. One-way and two-way ANOVA were applied to the results of spawning quality parameters (total eggs; fertilized eggs; viable eggs; hatched larvae; 3dph larvae per spawn per kg female and fertilization, egg viability, hatching, and larval survival rates), egg biochemical and fatty acid composition of experimental diet feeding phase to determine the combined effects of broodstock selection (HG or LG) and diet (FO or RO) and interaction of broodstock selection and diet. All data were analysed using the program IBM SPSS version 20 for Windows (IBM SPSS Inc., Armonk, NY, USA).

6.3 Results

6.3.1 Phase-I: Evaluation of mass spawning quality before feeding the experimental diet

6.3.1.1 Egg and larval quality

After one month of feeding the commercial diet, at the beginning of the spawning season and prior to feeding the experimental diets the spawning quality was evaluated, it was observed that the total number of eggs and fertilized eggs per spawn per kg female were

significantly (P<0.05) higher for low growth trait broodstock (LG) than for high growth trait broodstock (HG) (Table 6-4). However, the number of viable eggs and hatched-out larvae were not significantly (P>0.05) different between HG or LG broodstock (Table 6-4). Moreover, the HG broodstock group produced a 67% higher number of 3 dph larvae per spawn per kg female (7079 larvae) compared to LG (4736 larvae) broodstock group (Table 6-4). Regarding the spawning quality parameters (Table 6-5), despite the egg fertilization rate did not (P>0.05) vary between broodstock groups, the percentage of viable eggs, hatching rate and larval survival (3 dph) were significantly (P<0.05) higher in the HG broodstock group as compared to LG broodstock (Table 6-5).

Table 6-4. Reproductive performance of gilthead seabream broodstock selected for high (HG) or low (LG) growth before experimental diet feeding period (n=18).

Fecundity parameters	High growth (HG)	Low growth (LG)	*t*-test (*p* Value)
Total number of eggs/spawn/kg female	20841^{b}±10840	30293^{a}±9599	0.009
Fertilized eggs/spawn/kg female	20683^{b}±10735	29986^{a}±9515	0.009
Viable eggs/spawn/kg female	11359±6699	11701±4690	0.860
Hatched larvae/spawn/kg female	10380±6026	9214±3810	0.493
Larval survival 3dph/spawn/kg female	7079±5108	4736±2381	0.091

Means bearing different superscript letters in each row differ significantly (p<0.05, Independent sample t-test).

Table 6-5. Spawning quality (%) of gilthead seabream broodstock selected for high (HG) or low (LG) growth before experimental diet feeding period (n=18).

Spawning quality parameters (%)	High growth (HG)	Low growth (LG)	*t*-test (*p* Value)
Fertilization %	99.3±0.5	99.0±0.7	0.172
Egg viability %	53.8^{a}±18.1	40.1^{b}±14.9	0.018
Hatching %	91.7^{a}±4.5	79.5^{b}±11.3	0.001
Larval survival (3 dph) %	67.4^{a}±18.5	51.6^{b}±12.3	0.005

Means bearing different superscript letters in each row differ significantly (p<0.05, Independent sample t-test).

6.3.1.2 Egg biochemical analysis

The chemical composition of eggs from either HG or LG broodstock before feeding the experimental diet did not show any significant (P>0.05) difference (Table 6-6). Crude protein and crude lipid contents of eggs from HG or LG broodstock were 72.3 % or 70.1 % and 19.3 % or 18.3 % of dry matter, respectively (Table 6-6). Fatty acid profiles of eggs from high or low growth broodstock were very similar, only some derivatives from desaturases such as

18:2n-9, or 18:3n-6, were significantly (P<0.05) reduced in HG broodstock, whereas the elongase products 20:1n-7 and 20:2n-6 were increased (Table 6-7). Besides, monoenes were also reduced (P<0.05) in eggs from L broodstock, mainly due to the reduction in 18:1n-9 and 22:1n-7 (Table 6-7). Finally, the ratio EPA/ARA was mildly increased (P<0.1) (Table 6-7).

Table 6-6. Biochemical composition of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth before feeding the broodstock experimental diets (n=3).

Egg biochemical composition	High growth (HG)	Low growth (LG)	*t*-test (*p* Value)
Crude protein (% DM)	72.3±5.7	70.1±3.9	0.545
Crude lipid (% DM)	19.3±1.0	18.3±0.4	0.131
Ash (% DM)	5.8±1.9	6.2±1.3	0.709

Means bearing different superscript letters in each row differ significantly (p<0.05, Independent sample *t*-test).

Table 6-7. Fatty acid composition (% total fatty acids) of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth before feeding the broodstock experimental diets (n=2).

Fatty acids (%TFA)	High growth (HG)	Low growth (LG)	*t*-test (*p* Value)
14:0	1.43±0.12^{a}	1.27±0.11^{b}	0.033
14:1n-5	0.06±0.01	0.05±0.01	0.156
15:0	0.2±0.01	0.19±0.02	0.097
15:1n-5	0.02±0^{a}	0.01±0^{b}	0.004
16:0 ISO	0.04±0	0.04±0	1.000
16:0	13.86±0.32	13.75±0.71	0.738
16:1n-7	3.2±0.23	3.02±0.22	0.199
16:1n-5	0.07±0.01	0.06±0.01	0.401
16:2n-4	0.12±0.02	0.11±0.02	0.210
17:0	0.12±0.01	0.11±0.01	0.292
16:3n-4	0.23±0.01^{a}	0.20±0.02^{b}	0.032
16:3n-3	0.08±0.01	0.07±0.01	0.687
16:4n-3	0.06±0.01	0.06±0.01	0.401
18:0	4.39±0.06	4.46±0.1	0.152
18:1n-9	24.18±0.64	23.74±0.57	0.234
18:1n-7	2.43±0.08	2.38±0.07	0.240
18:1n-5	0.13±0	0.12±0.01	0.092
18:2n-9	0.22±0.03^{b}	0.29±0.02^{a}	0.001
18:2n-6	9.95±0.22	9.68±0.26	0.091
18:2n-4	0.12±0.01	0.11±0.02	0.341
18:3n-6	0.26±0.02^{b}	0.33±0.01^{a}	0.001
18:3n-4	0.16±0.02	0.15±0.02	0.348
18:3n-3	2.15±0.07	2.12±0.14	0.642

18:3n-1	0.01±0.01	0.01±0	0.549
18:4n-3	0.41±0.04	0.43±0.03	0.344
18:4n-1	0.09±0.01	0.08±0.01	0.263
20:0	0.06±0	0.06±0	1.000
20:1n-9	0.12±0	0.12±0.01	0.203
20:1n-7	0.73±0.01[a]	0.69±0.03[b]	0.004
20:1n-5	0.1±0	0.1±0.01	0.175
20:2n-9	0.11±0.02	0.1±0.01	0.532
20:2n-6	0.38±0.01[a]	0.35±0.01[b]	0.001
20:3n-9	0.03±0.01	0.03±0.01	0.599
20:3n-6	0.26±0.03	0.26±0.01	0.888
20:4n-6	0.88±0.05	0.87±0.07	0.781
20:3n-3	0.31±0.03	0.29±0.01	0.086
20:4n-3	0.79±0.01	0.76±0.04	0.074
20:5n-3	5.2±0.34	5.26±0.4	0.789
22:1n-11	0.15±0.01	0.14±0.01	0.183
22:1n-9	0.08±0.01	0.08±0.01	0.599
22:4n-6	0.08±0	0.08±0.01	0.363
22:5n-6	0.27±0	0.26±0.02	0.127
22:5n-3	2.75±0.09	2.66±0.12	0.207
22:6n-3	23.65±1.16	24.97±1.14	0.076
Total Saturates	20.06±0.47	19.84±0.79	0.575
Total Monoenes	31.28±0.63[a]	30.52±0.53[b]	0.048
Total n-3	35.41±1.13	36.63±1.28	0.110
Total n-6	12.08±0.22	11.83±0.23	0.076
Total n-9	24.71±0.66	24.33±0.58	0.311
Sum n-3 LC-PUFA	32.71±1.15	33.94±1.3	0.112
EPA/ARA	5.93±0.13	6.08±0.12	0.070
ARA/EPA	0.17±0	0.17±0.01	0.260
DHA/EPA	4.57±0.44	4.77±0.42	0.426
DHA/ARA	27.08±2.5	29.04±2.95	0.244
n-3/n-6	2.93±0.15	3.10±0.15	0.080

Means bearing different superscript letters in each row differ significantly (p<0.05, Independent sample t-test).

6.3.2 Phase-II: Evaluation of mass spawning quality after feeding the experimental diets

6.3.2.1 Egg and larval quality

The total number of eggs or fertilized eggs produced per spawn per kg female, was significantly (one-way ANOVA, P<0.01, Table 6-8) lowest for LGRO broodstock and highest for LGFO. Thus, in LG broodstock, feeding the RO diet instead of the FO diet reduced both the total number of eggs and the number of fertilized eggs, whereas such effect was not found in HG broodstock, reflecting the interaction between selection and diet in these two parameters

(two-way ANOVA, $P<0.01$). However, regarding the number of viable eggs, hatchlings, and 3 dph larvae per spawn per kg female, all these parameters were significantly improved by broodstock selection (two-way ANOVA, $P<0.01$), being increased in HGRO broodstock in comparison to LGRO (one-way ANOVA, $P<0.01$, Table 6-8). This effect was not observed in fish fed diet FO, reflecting the interaction between selection and diet in these three parameters (two-way ANOVA, $P<0.01$). Thus, the hatchling and 3 dph larvae production were respectively 38 and 35% higher in the HGRO broodstock compared to LGRO broodstock.

Regarding the spawning quality parameters, fertilization rates were improved in eggs from high growth broodstock (two-way ANOVA, $P<0.01$, Table 6-9) and fed the FO diet (two-way ANOVA, $P<0.05$, Table 6-9). Therefore, the combination of selection for high growth and feeding the FO diet resulted in significantly highest fertilization rates (one-way ANOVA, $P<0.01$). Similarly, the egg viability rate was significantly highest (86%) for HF broodstock followed by HGRO (74%) and LGRO broodstock (one-way ANOVA, $P<0.01$), and there was a positive significant effect of selection for high growth, regardless the diet fed (two-way ANOVA, $P<0.01$, Table 6-9). Besides, feeding the RO diet also had a significant effect on egg viability rates (two-way ANOVA, $P<0.01$, Table 6-9), which were reduced in HG fish and increased in LG fish, denoting the interaction between both factors diet and selection (two-way ANOVA, $P< 0.01$, Table 6-9). Likewise, selection for H growth significantly improved hatching rates (two-way ANOVA, $P<0.05$, Table 6-9) in broodstock fed FO diet, whereas this effect was not observed in fish fed RO diet, denoting the interaction between both factors (two-way ANOVA, $P<0.01$, Table 6-9). Thus, the lowest (one-way ANOVA, $P<0.001$) hatching rate (86.8%) was observed in LF broodstock. The 3dph larval survival rate was not significantly affected by selection or diet (Table 6-9).

Table 6-8. Reproductive performance of gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or RO diet over three months of reproductive months (n=25).

Fecundity parameters	HGFO	HGRO	LGFO	LGRO	One-way ANOVA (*p* Value)	Two-way ANOVA (*p* Values)		
						Selection	Diet	S x D
Total number of eggs/spawn/kg female	22897^{b}±10989	43176^{a}±16830	46047^{a}±18533	16459^{b}±8789	< 0.001	0.536	0.108	< 0.001
Fertilized eggs/spawn/kg female	22397^{b}±10756	41533^{a}±16305	44183^{a}±18118	15749^{b}±8661	< 0.001	0.477	0.100	< 0.001
Viable eggs/spawn/kg female	19560^{bc}±9141	31669^{a}±12425	22799^{ab}±18735	11528^{c}±7061	< 0.001	< 0.001	0.869	< 0.001
Hatched larvae/spawn/kg female	19141^{bc}±8950	29629^{a}±11882	19945^{b}±16647	11112^{c}±6884	< 0.001	< 0.001	0.724	< 0.001
3dph larvae/spawn/kg female	11649^{ab}±6627	16977^{a}±10024	10328^{bc}±7048	5924^{c}±4939	< 0.001	< 0.001	0.755	0.001

Means bearing different superscript letters in each row differ significantly (p<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 6-9. Spawning quality (%) of gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or RO diet over three months of reproductive months (n=25).

Spawning quality parameters (%)	HGFO	HGRO	LGFO	LGRO	One-way ANOVA (*p* Value)	Two-way ANOVA (*p* Values)		
						Selection	Diet	S x D
Fertilization %	97.7^{a}±1.0	96.0^{b}±1.8	95.8^{b}±1.8	95.5^{b}±3.6	0.003	0.007	0.030	0.132
Egg viability %	86.3^{a}±5.6	74.4^{b}±9.9	44.9^{c}±18.9	70.5^{b}±11.6	< 0.001	< 0.001	0.007	< 0.001
Hatching %	97.6^{a}±1.7	93.4^{a}±5.6	86.8^{b}±15.9	96.1^{a}±2.6	< 0.001	0.020	0.150	< 0.001
Larval survival (3 dph) %	61.4±21.1	58.3±21.4	55.6±15.0	51.4±20.4	0.326	0.108	0.357	0.880

Means bearing different superscript letters in each row differ significantly (p<0.05, One-way ANOVA, Tukey Post-Hoc).

6.3.2.2 Egg biochemical analysis

Crude protein and ash content did not differ among eggs from the different broodstock groups (Table 6-10). However, crude lipid contents of eggs from HF and LF group were significantly (P<0.001) higher than those from HGRO and LGRO groups, denoting the highly significant effect of RO diet.

Egg fatty acid composition markedly reflected the fatty acid profile of the broodstock diet (Table 6-11). Thus, feeding the rapeseed oil diet significantly reduced the egg contents in saturated fatty acids, such as 14:0, 15:0, 16:0, 17:0 or 18:0, and PUFA, such as 18:2n-4, 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, as well as total n-3, n-3 LC-PUFA and n-3/n-6 (Table 6-11). On the contrary, feeding the RO diet significantly increased the egg contents in monounsaturated and n-9 fatty acids, such as 18:1n-9, 20:1n-7 or 22:1n-9, in n-6 fatty acids, such as 18:2n-6, 20:2n-6, as well as 18:3n-3, DHA/EPA and DHA/ARA. The egg DHA content was not significantly different (P>0.05) among broodstock groups (Table 6-11). Two-way ANOVA analysis indicate that the egg fatty acid composition is altered by dietary fatty acid composition: fish oil based diet significantly (P<0.05) increased egg ARA and EPA contents but had only a relatively smaller (P>0.05) impact on DHA content although the total n-3 LC-PUFA contents and n-3/n-6 ratios were significantly (P<0.05) improved (7-12% and 40-51%, respectively) in the groups fed the fish oil based diet (Table 6-11). Selection for high growth significantly increased 18:1n-5, 18:3n-4, 18:3n-3, and the EPA/ARA ratio, as well as the elongase products 20:2n-6 and 20:3n-3, and reduced 18:0, 18:2n-4 and 22:1n-9.

Table 6-10. Biochemical composition of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or RO diet over three months of reproductive months (n=3).

Egg biochemical composition	HGFO	HGRO	LGFO	LGRO	One-way ANOVA (*p* Value)	Two-way ANOVA (*p* Values)		
						Selection	Diet	S x D
Crude protein (% DM)	65.3±1.7	64.0±2.1	65.2±0.9	64.3±0.8	0.675	0.889	0.256	0.801
Crude lipid (% DM)	24.4^{a}±0.4	17.6^{b}±0.2	24.6^{a}±0.3	17.5^{b}±0.4	0.001	0.743	0.000	0.580
Ash (% DM)	6.9±0.4	6.8±0.3	7.0±0.6	5.8±0.8	0.085	0.188	0.072	0.114

Means bearing different superscript letters in each row differ significantly (p<0.05, One-way ANOVA, Tukey Post-Hoc).

Table 6-11. Fatty acid composition (% total fatty acids) of eggs obtained from the gilthead seabream broodstock selected for high (HG) or low (LG) growth fed with either FO or RO diet over three months of reproductive months (n=2).

Fatty acids (%TFA)	HGFO	HGRO	LGFO	LGRO	One-way ANOVA *p* value	Two-way ANOVA (*p* Values)		
						Selection	Diet	S x D
14:0	2.45±0.37^{ab}	1.38±0.08^{c}	2.83±0.59^{a}	1.53±0.36^{bc}	0.005	0.272	0.001	0.620
14:1n-5	0.09±0.01^{a}	0.05^{b}	0.1±0.02^{a}	0.05±0.02^{b}	0.003	0.328	0.001	0.549
14:1n-7	0.01±0.01	0.01	0.02±0.01	0.01	0.219	0.500	0.067	0.500
15:0	0.27±0.02^{ab}	0.17±0.01^{c}	0.3±0.05^{a}	0.19±0.03^{bc}	0.003	0.206	0.001	0.659
15:1n-5	0.02	0.01±0.01	0.02±0.01	0.02±0.01	0.487	1.000	0.282	0.282
16:0 ISO	0.05±0.01^{a}	0.03±0.01^{b}	0.05±0.01^{a}	0.03±0.01^{b}	0.005	1.000	0.001	0.397
16:0	15.02±0.57^{ab}	11.21±0.17^{c}	16.36±2.08^{a}	12.51±1.42^{bc}	0.005	0.115	0.001	0.979
16:1n-7	4.96±0.33^{a}	2.62±0.13^{b}	5.63±0.77^{a}	2.74±0.31^{b}	0.001	0.165	0.001	0.323
16:1n-5	0.09±0.01	0.06±0.01	0.1±0.03	0.07±0.01	0.060	0.372	0.012	0.855
16:2n-4	0.36±0.02^{a}	0.16±0.02^{b}	0.42±0.05^{a}	0.16±0.02^{b}	0.001	0.116	0.001	0.158
17:0	0.31±0.02^{a}	0.13±0.01^{b}	0.33±0.03^{a}	0.14±0.01^{b}	0.001	0.174	0.001	0.397
16:3n-4	0.21±0.01^{ab}	0.17±0.01^{b}	0.23±0.04^{a}	0.17±0.02^{b}	0.018	0.617	0.003	0.457
16:3n-3	0.15±0.08	0.07	0.12±0.02	0.07±0.01	0.103	0.626	0.022	0.533
16:3n-1	0.06	0.06	0.08±0.02	0.05±0.01	0.129	0.446	0.098	0.098
16:4n-3	0.12±0.02^{a}	0.05±0^{b}	0.14±0.02^{a}	0.06±0.02^{b}	0.001	0.183	0.001	0.840

16:4n-1	0.01±0.01	0.01±0.01	0.01	0.01	0.596	0.195	1.000	1.000
18:0	3.9±0.07^{ab}	3.36±0.13^{b}	4.36±0.33^{a}	3.66±0.22^{b}	0.002	0.015	0.001	0.546
18:1n-9	18.13±0.25^{b}	29.01±1.23^{a}	16.67±1.51^{b}	30.78±2.16^{a}	0.001	0.860	0.001	0.091
18:1n-7	2.68±0.01	2.59±0.05	2.91±0.28	2.68±0.1	0.144	0.103	0.109	0.480
18:1n-5	0.16±0.01^{ab}	0.14^{b}	0.18±0.02^{a}	0.15±0.01^{b}	0.009	0.043	0.004	0.218
18:2n-9	0.16±0.01	0.17±0.01	0.15±0.02	0.18±0.01	0.039	0.631	0.008	0.347
18:2n-6	5.8±0.08^{b}	11.02±0.44^{a}	4.84±0.33^{b}	11.47±0.54^{a}	0.001	0.290	0.001	0.014
18:2n-4	0.19^{b}	0.1^{c}	0.23±0.02^{a}	0.09±0.01^{c}	0.001	0.043	0.001	0.008
18:3n-6	0.23±0.03	0.23±0.02	0.22±0.02	0.23±0.01	0.878	0.792	0.601	0.601
18:3n-4	0.27±0.02^{a}	0.16±0.01^{b}	0.25±0.02^{a}	0.12±0.03^{b}	0.001	0.023	0.001	0.247
18:3n-3	1.59±0.02^{b}	3.15±0.18^{a}	1.17±0.04^{c}	3.25±0.06^{a}	0.001	0.024	0.001	0.002
18:3n-1	0.02±0.01	0.01	0.02	0.01	0.045	0.631	0.008	0.631
18:4n-3	1.09±0.04^{a}	0.66±0.04^{b}	1.14±0.05^{a}	0.62±0.03^{b}	0.001	0.943	0.001	0.064
18:4n-1	0.15±0.04^{a}	0.08±0.02^{b}	0.18±0.01^{a}	0.08±0.01^{b}	0.001	0.211	0.001	0.308
20:0	0.07±0.01^{b}	0.09^{a}	0.08±0.01^{ab}	0.09±0.02^{ab}	0.041	0.524	0.010	0.219
20:1n-9	0.16^{ab}	0.14±0.01^{b}	0.18±0.01^{a}	0.14±0.01^{b}	0.001	0.076	0.001	0.076
20:1n-7	0.96±0.03^{c}	1.19±0.02^{a}	1.03±0.08^{bc}	1.14±0.06^{ab}	0.003	0.715	0.001	0.112
20:1n-5	0.14±0.01^{a}	0.12±0.01^{b}	0.15±0.01^{a}	0.11±0.01^{b}	0.001	0.500	0.001	0.195
20:2n-9	0.08^{a}	0.05^{b}	0.07±0.01^{a}	0.05^{b}	0.001	0.081	0.001	0.081
20:2n-6	0.23^{b}	0.3^{a}	0.21±0.02^{b}	0.29^{a}	0.001	0.016	0.001	0.471
20:3n-9	0.06±0.01^{a}	0.03^{b}	0.05±0.01^{a}	0.03^{b}	0.001	0.667	0.001	0.667
20:3n-6	0.16±0.01^{a}	0.13±0.01^{b}	0.16^{a}	0.13^{b}	0.001	0.195	0.001	1.000
20:4n-6	1.05±0.05^{a}	0.65±0.02^{b}	1.11±0.04^{a}	0.63±0.05^{b}	0.001	0.379	0.001	0.111
20:3n-3	0.21±0.01^{a}	0.22±0.01^{a}	0.18±0.01^{b}	0.22±0.01^{a}	0.002	0.010	0.002	0.029
20:4n-3	0.96±0.02^{a}	0.68±0.02^{b}	0.99±0.04^{a}	0.62±0.05^{b}	0.001	0.455	0.001	0.059
20:5n-3	9.33±0.22^{a}	5.65±0.19^{b}	9.57±0.92^{a}	5.24±0.74^{b}	0.001	0.825	0.001	0.378
22:1n-11	0.28±0.01^{b}	0.3±0.02^{ab}	0.32±0.02^{a}	0.27±0.01^{b}	0.017	0.565	0.347	0.003
22:1n-9	0.10±0.01^{b}	0.15±0.01^{a}	0.10±0.02^{b}	0.12±0.01^{ab}	0.001	0.043	0.001	0.098
22:4n-6	0.09±0.01^{a}	0.07±0.01^{b}	0.10±0.01^{a}	0.06±0.01^{b}	0.001	0.347	0.001	0.081
22:5n-6	0.27±0.01^{a}	0.21±0.01^{b}	0.29±0.04^{a}	0.18±0.02^{b}	0.001	0.696	0.001	0.078
22:5n-3	2.85±0.12^{a}	2.16±0.14^{ab}	2.95±0.5^{a}	1.77±0.33^{b}	0.005	0.439	0.001	0.213
22:6n-3	24.45±1.01	20.99±1.93	23.41±4.75	17.78±4.1	0.144	0.299	0.045	0.589

Total Saturates	22±0.92^{ab}	16.34±0.13^{c}	24.25±3.05^{a}	18.1±2.03^{bc}	0.003	0.104	0.001	0.829
Total Monoenes	27.77±0.4^{b}	36.38±1.43^{a}	27.39±2.71^{b}	38.29±2.66^{a}	0.001	0.535	0.001	0.360
Total n-3	40.76±1.31^{a}	33.64±2^{ab}	39.67±6.13^{ab}	29.63±5.18^{b}	0.036	0.323	0.008	0.563
Total n-6	7.84±0.07^{b}	12.61±0.41^{a}	6.95±0.29^{b}	13±0.47^{a}	0.001	0.239	0.001	0.012
Total n-9	18.62±0.25^{b}	29.53±1.24^{a}	17.16±1.55^{b}	31.27±2.16^{a}	0.001	0.872	0.001	0.096
Sum n-3 LC-PUFA	37.81±1.38^{a}	29.7±2.22^{ab}	37.11±6.16^{a}	25.63±5.23^{b}	0.021	0.358	0.004	0.512
EPA/ARA	8.93±0.15	8.77±0.07	8.61±0.54	8.33±0.51	0.321	0.120	0.347	0.803
ARA/EPA	0.11	0.11	0.12±0.01	0.12±0.01	0.099	0.028	0.397	0.397
DHA/EPA	2.62±0.05^{b}	3.71±0.22^{a}	2.43±0.29^{b}	3.36±0.33^{a}	0.001	0.092	0.001	0.596
DHA/ARA	23.4±0.09^{b}	32.54±1.8^{a}	20.99±3.56^{b}	28.13±4.37^{ab}	0.006	0.081	0.001	0.575
n-3/n-6	5.2±0.17^{a}	2.67±0.24^{b}	5.74±1.1^{a}	2.29±0.47^{b}	0.001	0.827	0.001	0.228

Means bearing different superscript letters in each row differ significantly (p<0.05, One-way ANOVA, Tukey Post-Hoc).

6.4 Discussion

The present study aimed to determine the impacts of broodstock selected for growth trait (HG or LG) fed with either a high or low marine ingredients (FO or RO) diet on the reproductive performance, egg quality, larval performance and egg biochemical and fatty acid compositions. Overall, broodstock selected for high growth (HG) and fed with a fish oil based diet (FO) showed the best spawning quality and egg fatty acid profiles.

Selective breeding programmes in gilthead seabream have addressed improvement of somatic or skeletal growth (Borrell et al., 2011; García-Celdrán et al., 2015; Lee-Montero et al., 2015; Lorenzo-Felipe et al., 2021), besides suggested impacts on intestinal functions (Perera et al. 2019). The effect of selection for improved growth on reproductive performance, growth trajectories, and intestinal plasticity have been reported for gilthead seabream (Ferosekhan et al., 2021; Perera et al., 2019). In the present study, we undertook mass spawning of gilthead seabream broodstock selected for either high (HG) growth or low (LG) growth and fed with diets containing two totally different fatty acid sources (FO or RO). The body weights of male and female from the high growth broodstock (HG) were significantly higher than those from the low growth broodstock (LG), denoting that the fish had been selected for growth. However, there was not any significant relation between fish growth and fecundity or spawning quality. The male and female broodstock ratio in this study was similar to the study conducted on gilthead seabream mass spawning for assessment of reproductive and genetic patterns of farmed gilthead seabream broodstock by microsatellite marker (Chavanne et al., 2014). The total broodstock number and biomass per broodstock group were also closely similar to other previous studies (Ferosekhan et al., 2021). The ingredients and proximate composition of either FO or RO diet used in this study were formulated in agreement to previous studies on nutritional programming (Izquierdo et al., 2015; Xu et al., 2019, Turkmen et al., 2020, Ferosekhan et al., 2020, 2021). The spawning period of gilthead seabream in the present study was observed from December to April in agreement to previous studies on this species (Fernández-Palacios et al., 1995; Izquierdo et al., 2015; Turkmen et al., 2020, Xu et al., 2019, Ferosekhan et al., 2020, 2021). The spawning quality parameters were in the range of previously reported for gilthead seabream broodstock (Scabini et al., 2011; Ferosekhan et al., 2020, 2021, Xu et al., 2019, Izquierdo et al., 2015).

In the present study, prior to feeding the experimental diets, despite the high number of total eggs produced / kg female/spawn in low growth broodstock, selection for high growth significantly improved egg viability, hatching and larval survival and lead to a higher number

of larvae. These results agree well with the increased viability and larval survival found in eggs from broodstock of fish selected for high growth in a previous study (Ferosekhan et al., 2021). In agreement, even after feeding the different experimental diets, selection for high growth increased the number of viable and hatched eggs and larval survival, particularly when fish were fed a diet with plant protein and oil sources. Even though fish selected for high growth had a larger body weight, no relation was found between the number of eggs and larvae produced and fish weight (R^2=0.1902), in agreement with previous studies (Ferosekhan et al., 2021). Thus, the results on fecundity and spawning parameters during both phases confirm the superior spawning quality of fish previously selected for high growth.

Besides, selection for high growth affected the egg fatty acid profiles, for instance reducing 18:0 and increasing 18:1n-5, 20:3n-3, 20:2n-6 and the EPA/ARA ratio, suggesting the modulation of lipid metabolism, which could be partially responsible for the improved spawning quality through a better utilization of dietary lipids. Moreover, a significant logarithmic regression could be observed between egg viability and egg content in 20:3n-3 ($y=0.001x+0.15$, $R^2=0.64$). Similarly, a logarithmic regression could be observed between egg fertilization rates and egg content in EPA/ARA ($y=20.95 \ln(x)-87.01$, $R^2=0.70$). The essential fatty acids ARA and EPA are precursors of eicosanoids, locally acting hormones that regulate a long list of physiological processes, including several related to reproduction (Izquierdo and Koven, 2011). EPA/ARA ratio is known to alter the production of eicosanoids (Wada et al., 2007; Fernández-Palacios et al., 2011), such as prostaglandins of the II and III series in gonads, what may affect a series of factors related to fertilization such as sperm production and quality, male sexual behavior and synchronizing spawning of the females and males (Sorensen et al., 1988; Fernández-Palacios et al., 2011). Thus, both ARA and EPA affect fertilization rates in gilthead seabream (Fernández-Palacios et al., 2005). Interestingly the egg DHA contents were not influenced by selection or diet, denoting the importance of this fatty acid and its selective retention in marine fish tissues (Koven et al., 1998; Izquierdo and Koven, 2011).

Comparison of the fatty acid profiles of the experimental diets showed that contents on LA and ALA, substrates for biosynthesis of ARA, EPA and DHA, were higher in RO diet, whereas the EPA and DHA were lower. Thus, dietary inclusion of RO increased LA and ALA levels, whereas inclusion of FO increased EPA and DHA. Accordingly, egg fatty acid profiles were affected by dietary lipids, although DHA levels were not significantly modified. Gilthead seabream has a high requirement for EPA and DHA during gametogenesis for the continuous supply of these EFAs to support the gamete development (Izquierdo et al., 2015, Ferosekhan

et al., 2020, 2021). Indeed, an inadequate supply of these EFAs has a deleterious effect on the spawning quality in fish (Izquierdo et al., 2001; Henrotte et al., 2010; Luo et al., 2015). In the present study, feeding the RO diet did not significantly affected fecundity parameters, which were in the range of those previously reported (Xu et al., 2019, Izquierdo et al., 2015, Ferosekhan et al., 2020), suggesting that the levels of dietary essential fatty acids fulfill the dietary requirements of gilthead seabream broodstock (Izquierdo et al., 2001; Fernández-Palacios et al., 2011). In contrast, in a previous study with the same broodstock, feeding a diet with RO and LO caused a 30-40% reduction in viable eggs, hatched eggs and 3-day-old larvae in comparison to fish fed the FO diet (Ferosekhan et al., 2021). Besides, egg viability, hatching and larval survival rates were reduced by 5-25% in broodstock fed the LO and RO diet comparison to the broodstock fed the FO diet (Ferosekhan et al., 2021). The content in LO, and subsequently in ALA, was the main difference between the diets used in the previous study (9.6 % ALA in total fatty acids) and in the present one (4.9% ALA in total fatty acids). Thus, although in the present study egg fatty acid profiles reflected dietary fatty acids contents, only ALA, LA and oleic acid (18:1n-9) markedly increased by 130%, 111% and 70%, respectively, in eggs from broodstock fed the RO diet in comparison broodstock fed the FO diet. In contrast, in the previous study, the egg contents in ALA increased by 257% in comparison to broodstock fed FO diet (Ferosekhan et al., 2021). Therefore, in comparison with the previous trial, the good reproductive performance of broodstock fed the RO diet together with the lower increase in the ALA in the present trial, suggest the deleterious effect of a diet high in ALA on reproductive performance of gilthead seabream broodstock. This hypothesis agree well with the alterations in steroid hormones production and release found in seabream fed diets high in LO (Montero et al., 2011) or the increase in cortisol release by seabream head kidney cells superfused with ALA (Ganga et al., 2011). In agreement, in our previous trial, feeding broodstock with a diet high in ALA, significantly altered steroid hormones production in females (Ferosekhan et al., 2021). Besides, the high ALA contents in diet and, subsequently, in tissues, also the ratio LA/ALA could be determinant of the negative effect of this fatty acid, since it may markedly affect lipid metabolism and steroid hormones (Yehuda et al., 2000). Thus, whereas in the present study the LA/ALA ratios were similar in eggs from broodstock fed FO or RO diets, in the previous study feeding the vegetable oil diet reduced LA/ALA ratios to half of those from broodstock fed the FO diet (Ferosekhan et al., 2021), what could be related to the reduction in egg viability, hatching and larval survival rates (Ferosekhan et al., 2021). In human platelets, incubation with ALA inhibits phospholipase A2, the key enzyme responsible for liberating the fatty acids that are eicosanoid precursors from the membrane phospholipids

(Ballou and Cheung, 1985). The modulation of lipid metabolism and stress physiology by ALA is mediated by protein kinases, which in turn affect AMP activation (Matthys and Widmaier, 1998).

6.5 Conclusion

In summary, the present study has shown that, either feeding with a commercial diet or with experimental diets containing different lipid profiles, selection for high growth improves broodstock reproductive performance in terms of egg viability, hatching and larval survival. Besides, selection for high growth affects the egg fatty acid profiles, particularly reducing the egg EPA/ARA ratios that were inversely related to broodstock performance. Feeding the RO diet did not negatively affected the fecundity parameters, nor egg DHA contents, denoting a sufficient content in essential fatty acids for gilthead seabream. Moreover, egg ALA contents were only moderately increased by feeding the RO diet, what probably contributed to avoid the important negative effects observed in previous studies. The present findings strongly suggest that broodstock selected for high growth trait has a positive effect on the improvement of egg and larval quality to produce quality offsprings and that feeding a RO diet does not negatively affect reproduction providing that fatty acid contents are sufficient for LC-PUFA and not excessive for ALA.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 (Blue Growth) H2020-BG-2018-818367 (AquaIMPACT project) and PROGENSA III (Mejora de la competitividad del sector de la dorada a través de la selección genética, JACUMAR program with support from the European Maritime and Fisheries). The first author gratefully acknowledges the financial assistance provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India for the doctoral study. This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein.

Authors' contribution

Conceived and designed the experiment: MI DM SF SS SK. Broodstock selection: MI DM SS SF JMA MJZ. Diet formulation and preparation: MI RF SF DM SK. Egg and larval quality evaluation: SS SF. Biochemical analysis SS SF MJZ. Data analysis: SF MI SK. Wrote the paper: SF SS MI SK DM.

CHAPTER 7

General Conclusions

Chapter 7

General Conclusions

1. The fatty acid biosynthesis process is regulated by fatty acyl desaturase (*fads*) and elongases. The *fads2* is considered as a rate-limiting factor in biosynthesis of n-3 and n-6 LC-PUFA in fish. The fatty acid synthesis takes place in the liver and those fatty acids mobilized to various organs. The study of *fads2* activity in liver is restricted because animals need to be sacrificed to collect the organ. In order to study the *fads2* activities and expression by a non-invasive method, the peripheral blood cells could be considered as the most suitable. Our study results indicated that the *fads2* expression levels in the PBCs and liver showed a significant positive correlation ($r = 0.89$; $P < 0.001$) in gilthead seabream broodstock. This finding has greater support in elucidating the *fads2* expression and FAs synthesis capacity of the broodstock to select fish with higher capacity to produce n-3LC-PUFA, EPA and DHA. It is concluded that PBC *fads2* can be utilized as a valid biomarker for fatty acid metabolism in fish and is applicable to broodstock selection programs. PBC *fads2* expression levels have a good potential as a non-invasive method to select animals having increased fatty acid bioconversion capability and a better ability to utilize fish meal and fish oil free diets (Chapter 3).

2. Based on the forementioned results (Chapter 3), we selected GSB broodstock with *fads2* as biomarker to improve the reproductive performance of GSB through nutritional programming approach. Broodstock selected for high fads2 expression and fed with rapeseed oil based diet (80% FO replaced) could produce better spawning quality than broodstock fed with FO diet and low *fads2* expressed group. Our study showed the feasibility to reduce dietary ARA, EPA and DHA content down to 0.43, 6.6 and 8.4% total fatty acids, respectively, in broodstock diets designed to induce nutritional programming effects in the offspring without affecting the egg and larval quality of GSB broodstock (Chapter 4).

3. The broodstock nutritional programming study in GSB through high *fads2* selection, facilitated higher EFA production in the eggs, which significantly improved the egg viability and larval survival rate (Chapter 4).

4. In Chapter 5, we studied the GSB reproductive performance based on the genetic selection for growth trait. In this study, we found that broodstock selected for high growth trait and fed with FO diet showed high performance in terms of fecundity, egg quality and larval production. It indicates that broodstock growth trait has positive influence on the spawning quality in gilthead seabream (Chapter 5).

5. The GSB mass spawning study was undertaken (Chapter 6) with an aim to know the effect of broodstock selection for high or low growth trait fed with either FO or RO diet on the reproductive performance of fish. The results showed that the broodstock selected for high growth trait has a strong positive effect on the reproductive performance of gilthead seabream broodstock fed with either rapeseed oil (HGRO) or fish oil (HGFO) based diet (Chapter 6).

The overall conclusion of the thesis

Fatty acyl desaturase 2 (*fads2*) gene expression in PBCs is an excellent biomarker to study the lipid and fatty acid metabolism in fish for improved growth and reproductive performance. Gilthead seabream broodstock selected for high *fads2* expression were able to produce high quality eggs and larvae even fed with low fish oil diet. Eggs obtained from high *fads2* broodstock showed higher expression of *fads2* during embryological development and had higher content of DHA, resulting in higher hatching and survival. In a similar way, broodstock selected for high growth trait showed strong positive effect on the improvement of egg and larval quality of gilthead seabream broodstock fed with either of FO or VO diets in the commercial scale mass spawning studies.

CHAPTER 8

RESUMEN AMPLIADO EN ESPAÑOL

Chapter 8

RESUMEN AMPLIADO EN ESPAÑOL

8.1 Introducción General

La acuicultura es el sector de la producción animal de más rápido crecimiento en el mundo, desempeña un papel importante en el suministro mundial de alimentos y se prevé que alcance los 377 mil millones de dólares para 2025 (FAO, 2020; AMR, 2021). El pescado no solo representa una excelente fuente de proteínas, aminoácidos y minerales, sino que también es una fuente única y rica de ácidos grasos poliinsaturados de cadena larga n-3 (n-3 LC-PUFA), especialmente ácido eicosapentaenoico (EPA; 20: 5n-3) y ácido docosahexaenoico (DHA; 22: 6n-3), y ambos AG son componentes dietéticos clave para la salud humana. Estos AG n-3 tienen efectos beneficiosos sobre el desarrollo neural en niños pequeños y en la prevención de enfermedades cardiovasculares e inflamatorias, así como trastornos neurológicos (Gogus y Smith, 2010). La demanda cada vez mayor de productos del mar ha dado lugar a un cambio de la pesca convencional a la producción intensiva de pescado (FAO, 2020). Dicha intensificación debe ser a largo plazo y tener mínimas consecuencias negativas para el medio ambiente y los recursos naturales (Klinger y Naylor, 2012). A lo largo de los años, ha aumentado la conciencia de la importancia de incluir recursos de ingredientes de piensos crudos sostenibles en los piensos para peces. La reducción en el nivel de inclusión de harina de pescado (FM) y aceite de pescado (FO) procedente de la pesca de captura se ha convertido en una práctica estándar durante la última década para garantizar que la acuicultura sea un sector de producción de alimentos sostenible y respetuoso con el medio ambiente para proporcionar nutrientes. dieta rica para consumo humano. Existe una presión continua sobre la industria de la acuicultura para reducir su dependencia de ingredientes de origen marino como FM y FO (Welch et al., 2010). Ambos ingredientes se han vuelto escasos en los últimos años y se prevé que los precios, que han subido constantemente durante las últimas tres décadas, aumentarán aún más a medida que la demanda siga aumentando. Como resultado, una reducción en el uso de MF y FO es una gran necesidad para el desarrollo de la acuicultura sostenible. Se reconoce que la incorporación de productos vegetales en altos niveles en la dieta de los peces tiene algunas desventajas, particularmente relacionadas con las diferencias y deficiencias de pocos o seleccionados aminoácidos (AA), AG y composición de minerales en comparación con los ingredientes marinos, pero también a la presencia de factores anti-nutricionales en ingredientes vegetales.

En consecuencia, los niveles de sustitución de los ingredientes marinos deben adaptarse según la especie y las etapas de desarrollo de los peces. Estas diferencias en la composición de los ingredientes vegetales pueden tener consecuencias metabólicas y fisiológicas que merecen una mayor investigación para proporcionar antecedentes adecuados para un mayor uso exitoso de los alimentos para plantas en alimentos acuícolas (Gatlin et al., 2007).

Se ha avanzado mucho en los últimos años en el reemplazo parcial de FM y FO por ingredientes vegetales en dietas nutricionalmente bien balanceadas (Oliva-Teles et al., 2015; Tacon et al., 2015). Se han reportado muchos estudios para el reemplazo completo de la FM por fuentes alternativas de proteína vegetal en las dietas para peces de agua dulce y marinos (Tacon et al., 2015; Kaushik et al., 2004; Torrecillas et al., 2017) y el reemplazo completo de la FM se logró en gran medida para los peces. Sin embargo, el reemplazo total de FO en las dietas de los peces marinos es difícil debido a la escasa o nula disponibilidad de n-3 LC-PUFA, que es muy esencial para los teleósteos marinos (Izquierdo, 1996; Tocher et al., 2010). El FO es una fuente naturalmente rica en n-3 LC-PUFA, a pesar de algunas diferencias en los perfiles de AG de los diferentes aceites de pescado. La disponibilidad mundial de "aceites de pescado" es muy limitada y, por lo tanto, debe utilizarse de manera sostenible. Los aceites vegetales (VO) se han utilizado para reemplazar parcialmente el FO, ya sea solo (Torstensen et al., 2008) o en combinación con el reemplazo de FM (Liang et al., 2014; Simó-Mirabet et al., 2018). El reemplazo de FO por VO está limitado por la falta de n-3 LC-PUFA en VO, a pesar de sus altas concentraciones en precursores de AG de 18 carbonos (18C), porque el teleósteo marino tiene una capacidad limitada de bioconversión de AG 18C en LC-PUFA como EPA y DHA. El paso inicial en la biosíntesis de LC-PUFA en los peces es catalizado por la enzima delta 6 desaturasa del ácido graso (Δ6 Fads), que inserta un doble enlace adicional en los precursores del 18C, ácido linoleico (LA, 18: 2n-6) o alfa-linolénico. ácido (ALA, 18: 3n-3). Por tanto, esta enzima produce 18: 3n-6 y 18: 4n-3 y, posteriormente, AG de cadena carbonada más larga y AG esenciales (EFA), como EPA (20: 5n-3) y DHA (22: 6n -3) se producen en peces después de varios pasos de elongación, desaturación y β-oxidación (Monroig et al., 2011; Tocher et al., 2015). Los teleósteos marinos tienen una baja expresión de acido graso desaturasa 2 (*fads2*), el gen que codifica la enzima Δ6 Fads (Tocher et al., 2015) y por lo tanto, n-3 y n-6 LC-PUFA deben incluirse en la dieta.

8.2 Potencial de producción de biología y acuicultura de la dorada

La dorada es un teleósteo marino que pertenece a la familia Sparidae que consta de gran cantidad de especies con varios géneros. Los espáridos tienen una amplia distribución

geográfica y se encuentran en los mares tropicales y templados y también se encuentran en aguas frías y salobres. La dorada (Figura 1-1) tiene forma de cuerpo ovalado, cuerpo profundo y comprimido, con una cabeza curva con ojos pequeños. Es una especie bentopelágica, que se encuentra en la costa y ambientes costeros, que habita en fondos arenosos y rocosos con lechos de pastos marinos y se reporta en profundidades de hasta 150 my es una especie de pez formadora de eurihalina y cardumen (Sola et al., 2006). Es principalmente una especie carnívora, se alimenta de pequeños crustáceos, moluscos, peces, y también se alimenta de pastos y malezas, etc. La biología reproductiva de esta especie se reporta como hermafrodita proterándrica, es un macho funcional durante los primeros dos años y luego se puede invertir a hembra. El desove ocurre típicamente de diciembre a abril, cuando la temperatura del agua es de 13-17 ° C. La dorada se cultiva principalmente en jaulas marinas con densidades medias de 15-25 kg m3. El período de cultivo suele tardar entre 1,5 y 2 años en alcanzar los 400-600 g en condiciones de producción comercial. Los requerimientos nutricionales de los principales nutrientes para la dorada han sido completados recientemente y esta información es vital para la formulación y preparación de dietas comerciales para la dorada. En muchos estudios, se observa que la dorada crece bien con dietas formuladas y preparadas para contener entre un 45% y un 50% de proteína cruda y entre un 18% y un 22% de lípidos crudos. Como para la mayoría de los peces marinos, las principales materias primas utilizadas en los piensos son MF y FO, aunque las fuentes vegetales alternativas se utilizan cada vez más en los últimos días (Oliva-Teles, 2000; Gómez-Requeni et al., 2004). La producción total de la dorada en 2018 fue de más de 200 000 toneladas (FAO, 2020) (Figura 1-2). En 2016, más del 90% de la acuicultura de la dorada se llevó a cabo en solo 5 de los 20 países, siendo Turquía el principal productor (31%), seguido de Grecia (26%), Egipto (14%), Túnez (7%) y España (5%). Esta especie también se está cultivando en muchos otros países mediterráneos, incluidos Chipre, Francia, Portugal, Croacia, Malta, Albania, Bosnia, Libia, Argelia y Marruecos (FAO, 2020).

8.3 Reproducción de dorada

La dorada es una especie con ovarios sincrónicos agrupados y de reproducción estacional, y el desove tiene lugar entre noviembre y junio, lo que corresponde a una temperatura natural del agua de 13-19 ° C. Producen puestas diariamente, de entre 30.000 y 40.000 huevos por kg de peso corporal por día y por hembra durante un período de unos 3 meses. La dorada desova espontáneamente en cautiverio en condiciones ambientales y dietéticas óptimas (Izquierdo et al., 2015; Xu et al., 2019; Ferosekhan et al., 2020).

8.4 Mejora genética de la dorada

La aplicación de la investigación de mejoramiento genético en animales y plantas ha contribuido sustancialmente a mantener el crecimiento y mejorar la productividad (Gjedrem et al., 2012). Sin embargo, en comparación con los animales de granja terrestres, la aplicación de los principios de la genética cuantitativa en los peces ha sido limitada y es necesario considerar más factores. Se han desarrollado varios programas de cría de salmón atlántico (*Salmo salar*), trucha arcoíris (*Onchorhynchus mykiss*), tilapia (*Oreochromis niloticus*), bagre (*Ictalurus punctatus*), lubina (*Dicentrarchus labrax*), rodaballo (*Psetta maxima*) y dorada (Gjedrem, 1997; Afonso et al., 2012). La cría selectiva se puede practicar siguiendo diferentes métodos y enfoques, desde la selección en masa más simple hasta la selección de índices y la selección asistida por marcadores para la producción de descendientes con los rasgos deseados. Todos los métodos de selección empleados en peces utilizan principalmente el desove masivo para producir grandes cantidades de la progenie deseada (Chavanne et al., 2016; Jansen et al., 2017). El programa de desove masivo tiene ciertas limitaciones en el proceso de selección, ya que carece de técnicas para identificar a los padres de la descendencia individual para conocer a los padres con mejor desempeño. Para superar este problema en el desove masivo, los marcadores de microsatélites se utilizan como una herramienta poderosa para identificar y asignar la contribución y el rendimiento de los padres a través del rendimiento de las crías (Navarro et al., 2009; Lee-Montero et al., 2013).

8.5 Nutrición de los reproductores

La composición nutricional y la calidad de la dieta de los reproductores desempeñan un papel importante en la calidad de los huevos y las larvas de los peces, lo que respalda la producción de crías de mejor rendimiento (Bell et al., 1997; Bruce et al., 1999; Izquierdo et al., 2001). Las composiciones de ácidos grasos (AG) de la dieta de los reproductores tienen un papel importante para asegurar la reproducción exitosa y la supervivencia de la descendencia (Izquierdo et al., 2001). La importancia de la nutrición de los reproductores en el desarrollo de las gónadas, los espermatozoides, la calidad del huevo y el rendimiento larvario es ampliamente reconocida, y varios estudios se han centrado en definir las funciones de los nutrientes clave en la reproducción de la dorada (Fernandez-Palacios et al., 1995; Scabini et al., 2010). La composición total de AG de los huevos de pescado refleja la composición dietética de la dieta de los padres y los ácidos grasos esenciales (AGE) de la dieta, como específicamente EPA y DHA, y su función en el desarrollo de huevos y larvas está bien documentada (Watanabe et al.,

1984, pág. Fernandez-Palacios et al., 1995; Bell et al., 1997; Bruce et al., 1999; Izquierdo et al., 2001, 2015).

La importancia de los n-3 LC-PUFA de la dieta, como EPA y DHA, para los peces marinos y su necesidad de suministrar cantidades suficientes de estos AG para la producción exitosa de huevos y larvas. está bien documentado para muchas especies de peces marinos (Izquierdo et al., 2001; Watanabe y Vassallo-Agius, 2003). Los peces marinos contienen grandes cantidades de EPA y DHA en los fosfolípidos de sus membranas celulares y los niveles más bajos de EPA y DHA en las dietas afectarán fuertemente los diversos cambios fisiológicos y de comportamiento en los peces, especialmente en las especies de peces marinos y también estos niveles reducidos. de los AG esenciales pueden afectar el sistema endocrino de los peces, ya que se informó que los AG esenciales son un cofactor importante y precursores en la vía de la esteroidogénesis (Sargent et al., 2003; Wathes et al., 2007). Durante el proceso de gametogénesis, los LC-PUFA n-3 como EPA y DHA se incorporan preferentemente a los óvulos para el desarrollo normal del embrión (Bromage, 1995). Los AGE como EPA, DHA y ARA son reconocidos como los principales AG que mejoran la calidad del huevo y el desarrollo de la descendencia (Harel et al., 1994, Bruce et al., 1997, Tocher et al., 2005). La inclusión dietética de diferentes fuentes de aceite y su perfil de AG afectan el desempeño reproductivo en la dorada. El nivel dietético de AGE para los reproductores de dorada se proporciona en la Tabla 1-1. Un trabajo reciente de nuestro grupo de investigación con reproductores de dorada alimentados con dietas específicamente diseñadas ricas en EPA y DHA ha demostrado un mejor rendimiento reproductivo y producción de huevos y larvas de alta calidad (Ferosekhan et al., 2020; Xu et al., 2019; Izquierdo et al., 2015). En estos estudios, observamos una fuerte correlación entre los AG de la dieta y la composición de AG de huevo en la dorada. Indica claramente que los AG en la dieta tienen un papel muy positivo en la mejora de la composición de los AG del huevo, lo que finalmente aumenta la calidad del huevo y las larvas en la dorada.

En la tabla 1-1 se puede observar que el estudio realizado para la programación nutricional ha utilizado un mayor nivel de inclusión de ácido linoleíco y ácido linlénico como precursores para la biosíntesis de AGE (Ferosekhan et al., 2020; 2021; Xu et al., 2019; Turkmen et al., 2019; Izquierdo et al., 2015) pero en los estudios primeros se le dio importancia a EPA y DHA para la suplementación directa de EFAs para mejorar el desempeño reproductivo en la dorada. El desempeño reproductivo de la dorada a través del enfoque de programación nutricional ha mejorado significativamente la calidad de los huevos y las larvas, incluso con una inclusión reducida de FM y FO en las dietas de los reproductores. En nuestros estudios

recientes se observó que el reemplazo de FO mediante una mezcla o el uso solo de aceite de colza (RO) o aceite de linaza (LO) hasta en un 100% no ha afectado el rendimiento reproductivo y la calidad de la descendencia de la dorada a través del enfoque de programación nutricional del reproductores seleccionados ya sea por una alta expresión de *fads2* o un rasgo de alto crecimiento y alimentados con dietas VO o suministro parcial de FO en las dietas (Ferosekhan et al., 2020; 2021; Xu et al., 2019; Turkmen et al., 2019; Izquierdo et al., 2019). al., 2015). Los parámetros de calidad de desove informados de todos estos estudios estaban en el rango de información previamente publicada de reproductores de dorada (Scabini et al., 2010; Fernandez-Palacios et al., 1995).

8.6 Necesidades de lípidos y ácidos grasos esenciales en el pescado

Los lípidos son un grupo variado de compuestos orgánicos que se caracterizan por su insolubilidad en agua debido a su estructura química. Los lípidos se pueden clasificar ampliamente en dos grupos: lípidos polares y neutros. Los fosfolípidos (PL) constituyen la mayoría de los lípidos polares, mientras que los triglicéridos (TAG) constituyen la mayoría de los lípidos neutros. Los lípidos, junto con las proteínas y los carbohidratos, son los principales macronutrientes necesarios para proporcionar tanto nutrientes vitales para la generación de energía como componentes básicos para el crecimiento celular y tisular. Los ácidos grasos esenciales (AGE) deben ser suministrados por lípidos para que el pescado satisfaga sus requisitos de AG. Los AGE son necesarios para la función óptima del metabolismo celular, así como para mantener la integridad estructural de la membrana, la reproducción, la calidad de los gametos y la función inmunológica en los peces, y los peces marinos no los producen de forma independiente (Tocher, 2003; 2015).

Los AGE son componentes importantes de los fosfolípidos y son los componentes principales de las membranas celulares y participan en el transporte de lipoproteínas. Además, se sabe que algunos de estos EFA, como ARA y EPA, son precursores de una clase importante de moléculas de señalización denominadas docosanoides y se sabe que poseen propiedades antiinflamatorias y protectoras en la síntesis de prostaglandinas (Bell et al., 1986). En el pescado, la naturaleza precisa de los AGE y sus necesidades dietéticas absolutas son difíciles de determinar, y existe una gran variabilidad entre las especies, en particular entre los peces de agua dulce y marinos (Tocher, 2003). Esta diferencia está esencialmente relacionada con la capacidad de los peces para bioconvertir C18 FA en LC-PUFA y, por lo tanto, con la actividad distinta de dos clases de enzimas implicadas en este proceso de conversión: elongasas y desaturasas. Las elongasas son responsables de la condensación de FA activados con

malonilCoA en la vía de elongación de FA, mientras que las desaturasas introducen un doble enlace en la cadena de acilo graso en la posición C6 o C5 del grupo carboxilo (Tocher, 2003). Se considera que esta capacidad de elongación y desaturación es más eficaz en peces de agua dulce que en peces marinos. Las redes tróficas marinas se caracterizan generalmente por altos niveles de n-3 LC-PUFA (EPA y DHA), que provienen principalmente de la presencia de microalgas, diatomeas y plancton, mientras que estos AG no se encuentran mucho en las redes tróficas de agua dulce (Parzanini et al., 2020). Por otro lado, las redes tróficas de agua dulce son ricas en ALA y LA y, por lo tanto, la capacidad generalizada de los peces de agua dulce para convertir PUFA 18C en PUFA 20C y 22C biológicamente activos puede ser el resultado de una adaptación evolutiva y una alta presión evolutiva para mantener la capacidad de producir de forma endógena LC-PUFA en especies de agua dulce (Tocher, 2003).

8.7 Biosíntesis endógena de ácidos grasos y actividad de moda

Los AG suministrados por la dieta y los sintetizados endógenamente por el animal pueden, hasta cierto punto, bioconvertirse en AG con cadenas más largas o insaturadas. En los vertebrados, el hígado representa el sitio principal para la síntesis de FA a través del complejo FA sintetasa a través de varios pasos de desaturación, alargamiento y oxidación. El pescado, como es el caso de todos los organismos, puede sintetizar de novo los AG saturados 16: 0 (ácido palmítico) y 18: 0 (ácido mirístico) en diferentes proporciones, dependiendo de la especie (Sargent et al., 2002). Todos los peces son capaces de convertir estos dos FA sintetizados de novo en FA monoinsaturados (MUFA), como 18: 1n-9, mediante la acción de la Δ9-desaturasa. Sin embargo, los peces, así como los mamíferos y otros vertebrados, carecen tanto de la Δ12-desaturasa como de la Δ15-desaturasa, que son necesarias para convertir 18: 1n-9 en 18: 2n-6 y 18: 2n-6 en 18: 3n- 3, respectivamente (Tocher, 2003). Por lo tanto, tanto 18: 2n-6 como 18: 3n-3 son AGE y, en consecuencia, deben ser suministrados por la dieta (Sargent et al., 2002, Tocher, 2003). Estos dos AG deben convertirse en sus productos bioactivos LC-PUFA: ácido araquidónico (ARA) de la serie n-6 y EPA y DHA de la serie n-3. Como se mencionó anteriormente, la capacidad para convertir C18 PUFA en LC-PUFA varía dentro de las especies de peces, siendo alta en agua dulce y baja en especies de peces marinos (Sargent et al., 2002, Tocher, 2003). La síntesis de ARA a partir de su precursor C18 requiere un primer paso de Δ6-desaturación seguido de un alargamiento para producir 20: 3n-6, que luego se desatura a 20: 4n-6 por la acción de Δ5-desaturasa. La síntesis de ARA luego requiere pasos adicionales de elongación y desaturación de Δ6, seguidos de un paso de acortamiento de la cadena en el peroxisoma (Sargent et al., 2002).

8.8 Fuentes dietéticas de EFA

Los aceites de pescado son la principal fuente de omega-3 LC-PUFA beneficiosos para la salud (Tabla 1-2). Está bien informado que la producción de pescado de las pesquerías marinas permanecerá estática en la próxima década y afectará la producción y el uso de FO en la acuicultura (Sargent y Tacon, 1999; Tacon, 2004). Durante la última década, la producción mundial de FO se ha estancado y no se espera que aumente más allá de los niveles actuales (FAO, 2020). Existen numerosas fuentes de lípidos con un uso potencial en la alimentación de peces como sustitutos de los alimentos orgánicos como subproductos animales, VO, productos marinos de niveles tróficos inferiores y plantas transgénicas y el uso de ingredientes de origen vegetal como alternativas sostenibles a los aceites marinos en Los piensos para peces tienen un gran potencial. Específicamente, los ingredientes vegetales tienen una alta disponibilidad global, en comparación con el FO, y tienen propiedades nutricionales que pueden satisfacer en gran medida los requisitos nutricionales del pescado.

8.9 Sustitución del aceite de pescado por aceites vegetales

Durante las últimas dos décadas, se han realizado considerables esfuerzos de investigación para evaluar el reemplazo del FO en la dieta por una variedad de fuentes alternativas de lípidos, incluidos los VO (aceite de linaza, aceite de colza, …) y grasas de animales terrestres (Fountoulaki et al., 2009). Los VO son ricos en C18-PUFA pero carecen de los n-3 LC-PUFA que se encuentran en abundancia en el FO. Sin embargo, la capacidad de los peces marinos para sintetizar LC-PUFA n-3 y n-6, como EPA, DHA y ARA a partir de los precursores ALA y LA presentes en el aceite vegetal, es muy limitada y, por lo tanto, el suministro dietético de EPA, DHA y ARA es esencial (Tocher, 2005; Izquierdo, 1996). Los altos niveles de VO en las dietas inducen cambios en los perfiles de AG de la dieta, lo que puede afectar no solo el rendimiento del crecimiento sino también el estado de salud de los peces marinos (Torrecillas et al., 2017). El estudio del reemplazo de FM y FO se ha llevado a cabo en varios proyectos europeos (Figura 1-4). Por ejemplo, en el proyecto ARRAINA se consiguió reducir FM y FO de 50-75% y 15% a 5% y 2.5%, respectivamente (ARRAINA, 2018).

8.10 Limitaciones en la sustitución de FO en la dieta por VO en la reproducción de peces

Como se describe en la sección anterior, se ha reconocido desde hace mucho tiempo que la nutrición de los reproductores tiene efectos significativos sobre el desarrollo gonadal de los peces y la calidad de los huevos (Watanabe 1985; Izquierdo et al., 2001, 2015). Además,

entre los constituyentes nutricionales de las dietas de reproductores, los lípidos son el componente que más afecta la composición del huevo (Izquierdo et al., 2001, 2015) y, en particular, el contenido de AGE en la dieta (Watanabe et al., 1984). Una función importante de EPA y DHA es su participación en los procesos reproductivos, particularmente con respecto a la calidad del huevo y el desarrollo de la progenie (Bell et al., 1986, Harel et al., 1994, Fernández-Palacios et al., 1995, Izquierdo et al., 2001, 2015). De hecho, la deficiencia en el contenido de n-3 LC-PUFA de las dietas de los reproductores se ha identificado como un factor importante que afecta negativamente el desempeño reproductivo de los peces (Izquierdo et al., 2001). Por lo tanto, una de las principales consecuencias cuando se reemplaza el FO por aceite vegetal puede estar relacionada con los cambios en el perfil dietético de AGE de los reproductores, lo que a su vez puede afectar la deposición de estos AG en los óvulos, dando como resultado un desove y huevos de menor calidad y consecuentemente afectar desarrollo de la descendencia y supervivencia larvaria. También se sabe que los PUFAs afectan potencialmente la reproducción a través de la regulación de la producción de eicosanoides, particularmente prostaglandinas, que están involucradas en varios procesos reproductivos. Esto incluye la producción de hormonas esteroideas, el desarrollo gonadal y la liberación de espermatozoides y óvulos. El ácido graso ARA es el principal precursor de la producción de eicosanoides (PE2) en los peces y el uso de VO, que carecen de ARA, también podría afectar el rendimiento reproductivo de los peces (Tocher, 2003; Izquierdo et al., 2001; Bell et al., 1986).

8.11 Enfoque de programación nutricional en la nutrición de peces

El término "programación" fue introducido en la literatura científica por Lucas (1991) para describir el proceso por el cual la exposición a un estímulo o condición específicos, durante las etapas críticas del desarrollo, puede resultar en cambios permanentes en las estructuras somáticas, los sistemas fisiológicos y el estado metabólico. del organismo. La extensión de este concepto al campo de la nutrición temprana se conoce como "programación nutricional" y se ha estudiado ampliamente en modelos de mamíferos para comprender las consecuencias en la edad adulta de una nutrición alterada durante los períodos pre o posnatal (Burdge y Lillycrop, 2010). Los mecanismos involucrados en la regulación metabólica por eventos nutricionales tempranos aún no se comprenden completamente en muchas especies; en algunas especies se ha demostrado que la programación nutricional puede estar mediada por la metilación del ADN, la acetilación de histonas y/o los ARN no codificantes (Lillycrop y Burdge, 2012). Se ha observado en algunas especies que los cambios epigenéticos se transmiten

de forma hereditaria, lo que respalda en gran medida la evidencia de la transmisión vertical de las madres a sus descendientes de enfermedades metabolicas. Además, los nuevos datos sobre el papel de los nutrientes y sus niveles de disponibilidad como moduladores de los mecanismos epigenéticos muestran una lista cada vez mayor de componentes dietéticos implicados en la programación nutricional (Badeaux y Shi, 2013).

En el desarrollo de los peces, los momentos de mayor sensibilidad y plasticidad metabólica (ventanas epigenéticas) están restringidas a los períodos de embriogénesis y desarrollo larvario temprano, similar a los mamíferos. En un estudio sobre el lenguado (*Solea senegalensis*) se ha observado que el crecimiento muscular de las larvas en metamorfosis se vé afectado por la temperatura de cultivo de las larvas recién eclosionadas, mediante la metilación del promotor del gen de la miogenina (Campos et al., 2013). Así, la temperatura de cultivo más baja (15ºC) induce la metilación del promotor de la miogenina, que afecta la expresión del gen de la miogenina en el músculo ocasionando una reducción del crecimiento muscular. En el mismo marco ambiental, una investigación en lubina europea sobre el cambio de la proporción de sexos en respuesta a la temperatura, mostró que la exposición a altas temperaturas (21ºC) aumenta el nivel de metilación del promotor del gen de la aromatasa gonadal (*cyp19a*) tanto en machos como en hembras (Navarro-Martín et al., 2011). Como resultado, se reprimió la expresión del gen de la aromatasa y, mediante el silenciamiento de este gen, se promovió el desarrollo de los machos durante la diferenciación sexual. Por lo tanto, existe una fuerte evidencia de que la determinación del sexo dependiente de la temperatura en la lubina europea puede controlarse mediante mecanismos epigenéticos de metilación del ADN (Navarro-Martín et al., 2011). Se han realizado otros estudios en diferentes especies de peces para esclarecer el papel de los factores ambientales como moduladores de los mecanismos epigenéticos durante la diferenciación y determinación de los fenotipos sexuales (Piferrer et al., 2012).

El estímulo nutricional durante las primeras etapas de la vida de los peces puede alterar el rendimiento en una etapa posterior y esto se puede lograr a través de varios enfoques, como a) la nutrición de los reproductores, a través de la transferencia de nutrientes a las reservas de el saco vitelino o la programación génica de los gametos y b) el inicio de la alimentación exógena, cuando la nutrición de las larvas puede ser manipulada por los primeros alimentos, ya sean enriquecimientos de presas vivas o dietas inertes. En la dorada, los efectos positivos de alimentar a los reproductores con una dieta enriquecida con AGE, se reflejaron en una mejor calidad de huevo y desarrollo larvario (Izquierdo et al., 2015; Turkmen et al., 2019; Xu et al., 2019; Ferosekhan et al., 2020). Recientemente, un estudio en la dorada reveló que la

programación nutricional temprana podría lograrse mediante la alimentación de los padres, con cambios metabólicos positivos a largo plazo en la descendencia. Cuando los reproductores se alimentaron con una dieta rica en aceite de linaza (más del 60% de reemplazo de FO), la progenie resultante demostró estar mejor adaptada para usar dietas con alto contenido de VO / proteínas vegetales, al mostrar una mejor tasa de crecimiento y utilización del alimento. Sin embargo, se encontraron algunos efectos negativos a corto plazo cuando el FO fue reemplazado al 100% por aceite de linaza en las dietas de reproductores, con fecundidad, calidad de desove, crecimiento y expresión del gen que codifica la Δ6 desaturasa (*fads2*) en postlarvas de 45DAH (Izquierdo et al., 2015).

8.12 Objetivos

La hipótesis principal de esta tesis es que "es posible programar nutricionalmente los alevines de dorada para una mejor utilización de dietas bajas en FO y FM mediante estimulos nutricionales de los reproductores y aplicar criterios de selección para crecimiento o mayor expresión de *fads2* sin afectar negativamente la calidad de la reproducción". Por ello, uno de los objetivos principales de los estudios realizados como parte de esta tesis fue generar conocimientos novedosos sobre el concepto de programación nutricional a través de la alimentación de reproductores de dorada. Además, se estudió el valor del uso de reproductores con mayor expresión de *fads2* como un biomarcador potencial de la calidad reproductiva de la dorada y la capacidad de los reproductores para producir una progenie con mejor utilización de dietas bajas en FO y FM. Más aún, se trató de identificar un método no lesivo para determinar la capacidad de expresión *fads2* de los reproductores, determinando la posible correlación entre la expresión de *fads2* en las células de sangre periférica (PBC) y el hígado. Finalmente, se determinó el efecto de la selección de reproductores para el rasgo de crecimiento, en combinación con distintas dietas sobre el desempeño reproductivo de la dorada en condiciones de desove masivo. Por tanto, para la consecución de estos fines se abordaron los siguientes objetivos específicos:

8.12.1 Selección de reproductores basada en la expresión *fads2*

1. Estudiar la correlación de la expresión génica de la desaturasa de AG 2 (*fads2*) entre las células sanguíneas periféricas (PBCs) y el hígado como biomarcador potencial para la selección de reproductores (Capítulo 3).

2. Evaluar el efecto de la mayor o menor expresión de *fads2* en los reproductores y de los perfiles de AG en la dieta sobre el desempeño reproductivo y la calidad del huevo en la dorada *Sparus aurata* (Capítulo 4).

8.12.2 Selección de reproductores basada en el rasgo de crecimiento

3. Estudiar la selección genética de reproductores para el rasgo de crecimiento y alimentados con niveles dietéticos altos o bajos de n-3 LC-PUFA sobre el rendimiento reproductivo, los niveles de hormonas esteroides, los espermatozoides y la calidad del huevo de la dorada, *Sparus aurata* (Capítulo 5).

4. Determinar el efecto de los reproductores con características de alto o bajo crecimiento alimentados con una dieta a base de FO o de colza sobre la calidad de desove de la dorada, *Sparus aurata* en condiciones de desove masivo (Capítulo 6).

8.13 Resumen de los experimentos

8.13.1 La relación entre la expresión del gen de la desaturasa de ácidos grasos 2 en las células sanguíneas periféricas (PBC) y el hígado en reproductores de *Sparus aurata* alimentados con una dieta baja en n-3 LC-PUFA

El objetivo principal de este estudio fue dilucidar la relación entre el patrón de expresión del *fads2* en células de sangre periférica (PBC) e hígado de reproductores de dorada, *Sparus aurata,* con el fin de determinar el posible uso de expresión de *fads2* como un biomarcador potencial para la selección de reproductores. Esta selección podría utilizarse para programas de reproducción que tengan como objetivo mejorar la reproducción, la salud y el estado nutricional. Los reproductores dorada marcados con PIT (machos: 1,22 ± 0,20 kg, 44,8 ± 2 cm; hembras: 2,36 ± 0,64 kg, 55,1 cm) fueron alimentados con una dieta que contenía niveles bajos de FM y FO (EPA 2.5; DHA 1.7 y n-3 LC-PUFA 4.6% TFA) durante un mes. Después del período de alimentación, la expresión de *fads2* en los PBC y el hígado de los reproductores machos y hembras se correlacionaron de manera muy significativa (r = 0,89; P<0,001). Además, en reproductores machos, la expresión de *fads2* se correlacionó significativamente (P<0.05) con el contenido del hígado en 16: 0 (r = 0.95; P = 0.04) y saturados totales (r = 0.97; P = 0.03) así como en 20:3n – 6/20: 2n – 6 (r = 0,98; P = 0,02), un indicador de la relación producto / precursor de *fads2*. En general, encontramos una correlación positiva y significativa entre los niveles de expresión de *fads2* en los PBC y el hígado de los reproductores de la dorada. Los niveles de expresión de PBC *fads2* indican un gran potencial

de utilización como método no invasivo para seleccionar animales que tienen una mayor capacidad de bioconversión de AG, más capaces de utilizar dietas bajas en FM y FO.

8.13.2 Rendimiento reproductivo de reproductores de dorada (*Sparus aurata*) con distinta expresión del gen de la desaturasa de ácidos grasos 2 y alimentados con dos perfiles de ácidos grasos en la dieta

Estudios anteriores han demostrado que es posible programar nutricionalmente las crías de dorada a través del reemplazo del FO por VO en la dieta de los reproductores, para mejorar su capacidad de crecimiento rápido cuando se alimentan con dietas bajas en FM y FO durante la fase de crecimiento. Sin embargo, en esos estudios el rendimiento de los reproductores se redujo por la dieta que contenía VO. En el presente estudio se modificaron los componentes de la dieta con VO para determinar si es posible reemplazar FO por una mezcla de FO y aceite de colza (RO), sin alterar el desempeño reproductivo de la dorada. Además, el estudio también tuvo como objetivo evaluar el desempeño reproductivo de reproductores con diferente expresión del gen de ácido graso desaturasa 2 (*fads2*), una enzima clave en la síntesis de AG poliinsaturados de cadena larga. Para ese propósito, los reproductores con una expresión alta (HD) o baja (LD) de *fads2* fueron alimentados durante tres meses de la temporada de desove con dos dietas que contenían diferentes perfiles de AG y se estudiaron sus efectos sobre las hormonas reproductivas, la fecundidad, el esperma y la calidad del huevo, la composición bioquímica del huevo y la expresión de *fads2*. Los resultados mostraron que la expresión de *fads2* en sangre en las hembras, que tendía a ser más alta que en los machos, se relacionaba positivamente con los niveles plasmáticos de 17β-estradiol. Además, los reproductores con alta expresión de *fads2* en sangre mostraron un mejor desempeño reproductivo, en términos de fecundidad y calidad del esperma y del huevo, lo que se correlacionó con la expresión de *fads2* de las hembras. Nuestros datos también mostraron que es factible reducir ARA, EPA y DHA a 0.43, 6.6 y 8.4% de AG totales, respectivamente, en dietas de reproductores diseñadas para inducir efectos de programación nutricional en la descendencia sin producir efectos adversos sobre la calidad del desove.

8.13.3 Influencia de la selección genética para el crecimiento, y los niveles dietéticos de n-3 LC-PUFA de la dieta de los reproductores en el rendimiento reproductivo de la dorada, *Sparus aurata*

La selección genética en la dorada, *Sparus aurata* se ha llevado a cabo para mejorar el crecimiento, la eficiencia alimenticia, la calidad del filete, las deformidades esqueléticas y

la resistencia a las enfermedades, pero no hay ningún estudio disponible para definir el efecto de la selección genética para el rasgo de crecimiento en el desempeño reproductivo de la dorada bajo condiciones de desove masivo. En este estudio, se seleccionaron reproductores la dorada de alto crecimiento (HG) o bajo crecimiento (LG) para evaluar las hormonas esteroides sexuales, el esperma, la calidad del huevo y el rendimiento reproductivo bajo diferentes regímenes de alimentación de una dieta comercial o una dieta experimental de reproductores que contiene FO o una dieta basada en aceite vegetal (VO). En la fase de alimentación con dieta comercial, los reproductores seleccionados para un crecimiento alto o bajo mostraron que el porcentaje de viabilidad del huevo mejoró positivamente (P = 0.014) en los reproductores con alto crecimiento. Los resultados de la alimentación con dieta experimental revelaron que tanto el rasgo de crecimiento como la composición de AG de la dieta influyeron en el rendimiento reproductivo de los reproductores la dorada. En la fase de alimentación de la dieta experimental, observamos que el rasgo de alto crecimiento masculino la dorada produjo un mayor número (P<0,001) y una mayor motilidad (P = 0,048) de los espermatozoides. La producción de huevos y larvas por puesta por kg de hembra mejoró significativamente en los reproductores de alto crecimiento y alimentados con una dieta a base de FO. Los resultados del presente estudio demuestran que los reproductores de dorada seleccionados en función del rasgo de crecimiento mejoran la calidad de los espermatozoides y los óvulos para producir una progenie viable.

8.13.4 La selección para un alto crecimiento mejora el rendimiento reproductivo de la dorada *Sparus aurata* en condiciones de desove masivo, independientemente de la fuente de lípidos de la dieta

Los programas de selección genética de la dorada se centran principalmente en los rasgos relacionados con el crecimiento, la resistencia a las enfermedades, las anomalías esqueléticas o la calidad del filete. Sin embargo, el efecto de la selección para el crecimiento sobre el rendimiento reproductivo de los reproductores de dorada no ha recibido mucha atención. Además, con el fin de programar nutricionalmente la dorada para una mejor utilización de dietas bajas en FM y FO, los reproductores se alimentan con dietas que contienen VO, que pueden afectar egativamente el rendimiento de los reproductores. El presente estudio tuvo como objetivo determinar el efecto de la selección por características de crecimiento, crecimiento alto (HG) o bajo (LG) y alimentación de reproductores con FO (dieta FO) o aceite de colza (dieta RO) como principales fuentes de lípidos, sobre el rendimiento reproductivo. de dorada. Durante la primera parte de la temporada de desove (Fase I), los reproductores HG y

LG se alimentaron con una dieta comercial y se observó que los reproductores HG produjeron un mayor número de larvas y mayores tasas de huevos viables, eclosión y supervivencia larvaria que los reproductores LG. En la Fase II los reproductores fueron alimentados con una de las dos dietas que contenían FO o RO. La fecundidad en términos de huevos viables, crías y larvas producidas, así como las tasas de fertilización, mejoraron en los reproductores HG. Algunos AG como 18: 0, 20: 2n-6, 20: 3n-3 o EPA/ARA también se vieron afectados por la selección de crecimiento. Según el ANOVA de 2 vías, la alimentación con la dieta RO no afectó significativamente los parámetros de fecundidad, pero redujo ligeramente las tasas de fertilización y eclosión en los reproductores HG. Sin embargo, los reproductores HG mostraron mejores parámetros de calidad de desove que los reproductores LG, incluso cuando fueron alimentados con la dieta RO. Los perfiles de AG del huevo reflejaron la composición de la dieta, aunque el contenido de DHA no se vio afectado. En conclusión, los reproductores seleccionados por su alto crecimiento tuvieron un efecto positivo en el rendimiento de los reproductores, y el reemplazo de FO por RO no afectó notablemente la reproducción, siempre que el contenido de AG fuera suficiente para satisfacer los requisitos de AG esenciales de los reproductores.

8.14 Conclusiones

1. El proceso de biosíntesis de AG está regulado por las desaturasas y elongasas de los AG, entre ellas, la Δ6 Fads se considera limitante. El hígado es el principal órgano de reconversión de los AG dietéticos y síntesis movilización a otros órganos. Los peces seleccionados por su mayor actividad Δ6 Fads tendrían una mayor capacidad para usar dietas en las que el FO, rico en LC-PUFA, fuera reemplazado por VO, ricos en sus precursores. El uso de la actividad de Δ6 Fads en el hígado está restringido porque los animales deben sacrificarse para recolectar el órgano. Para estudiar la actividad y expresión de *fads2* por un método no invasivo, las células sanguíneas periféricas podrían considerarse como las más adecuadas. Los resultados de nuestro estudio indican que los niveles de expresión de *fads2* en los PBC y el hígado están estrechamente relacionados en los reproductores de dorada. Este hallazgo favorece el uso de la expresión de *fads2* y la capacidad de síntesis de AG de los reproductores en la selección de peces con mayor capacidad para producir n-3LC-PUFA, EPA y DHA. Se concluye que la determinación de la expresión de la *fads2* en PBC puede utilizarse como un biomarcador válido aplicable a los programas de selección de reproductores. Además, los niveles de expresión de *fads2* en PBC tienen un buen potencial como

método no invasivo para seleccionar animales que tienen una mayor capacidad de bioconversión de AG y una mejor capacidad para utilizar dietas libres de harina y FO.

2. Los reproductores con una alta expresión de *fads2* y alimentados con una dieta basada en aceite de colza (80% de FO reemplazado) presentan mejor calidad de puestas y huevos que los alimentados con una dieta de FO y baja *fads2*. Además, es posible reducir el contenido de ARA, EPA y DHA en la dieta hasta 0.43, 6.6 y 8.4% del total de AG, respectivamente, en dietas de reproductores diseñadas para inducir efectos de programación nutricional en la descendencia, sin afectar la calidad de los huevos y las larvas de los reproductores de dorada.

3. Los reproductores de dorada con una alta expresión de *fads2* presentan una mayor producción de AGE en los huevos y una mejora significativa en la viabilidad del huevo y la tasa de supervivencia de las larvas.

4. Los reproductores seleccionados por sus características de alto crecimiento y alimentados con dieta FO presentan un alto rendimiento en términos de fecundidad, calidad del huevo y producción de larvas.

5. Los reproductores seleccionados por su alto crecimiento mejoran el desempeño reproductivo de los reproductores de dorada alimentados con una dieta basada en aceite de colza o FO.

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Appendices

Appendices

Publications, conference, training courses and webinar participation and other activities performed during PhD study period

1. Publications

a. Peer-reviewed articles (First authored PhD publications)

Ferosekhan, S., Turkmen, S., Xu, H., Afonso, J. M., Zamorano, M. J., Kaushik, S., and Izquierdo, M. 2020. 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*(7), 117. **(IF: 3.817), (Chapter-3).**

Ferosekhan, S., Xu, H., Turkmen, S., Gómez, A., Afonso, J. M., Fontanillas, R., and Izquierdo, M. 2020. Reproductive performance of gilthead seabream (*Sparus aurata*) broodstock showing different expression of fatty acyl desaturase 2 and fed two dietary fatty acid profiles. *Scientific reports*, 10(1), 1-14. **(IF: 4.379), (Chapter-4).**

Ferosekhan, S., Turkmen, S., Pérez-García, C., Xu, H., Gómez, A., Shamna, N., Afonso, J.M., Rosenlund, G., Fontanillas, R., Gracia, A. and Izquierdo, M., 2021. Influence of Genetic Selection for Growth and Broodstock Diet n-3 LC-PUFA Levels on Reproductive Performance of Gilthead Seabream, *Sparus aurata*. *Animals*, 11(2), 519. **(IF: 2.752), (Chapter-5).**

Ferosekhan, S., Sarih, S., Afonso, J.M., Zamorano, M.J., Fontanillas, R., Izquierdo, M., Kaushik, S., and Montero, D. 2022. Selection for high growth improves reproductive performance of gilthead seabream *Sparus aurata* under mass spawning conditions, regardless of the dietary lipid source. (Under review: *Animal Reproduction Science*), **(Chapter-6).**

b. Peer-reviewed articles (Second authored PhD publications)

Xu, H., **Ferosekhan, S.,** Turkmen, S., Afonso, J. M., Zamorano, M. J., & Izquierdo, M. (2020). Influence of parental fatty acid desaturase 2 (Fads2) expression and diet on gilthead seabream (*Sparus aurata*) offspring *fads2* expression during ontogenesis. *Animals*, *10*(11), 2191. **(IF: 2.752)**

Xu, H., **Ferosekhan, S.,** Turkmen, S., Afonso, J.M., Zamorano, M.J. and Izquierdo, M., 2021. 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. *Aquaculture*, 535, 736321. **(IF: 4.242)**

c. Other Peer-reviewed articles

Ferosekhan, S., Sahoo, S.K., Radhakrishnan, K., Gokulakrishnan, M., Giri, S.S., Pillai, B.R., and S.K. Swain. 2022. Standardization of weaning protocol for *Clarias magur* (Hamilton, 1822) larvae. *Asian Fisheries Science* (Accepted).

Ferosekhan, S., M. SriHari, K. Radhakrishnan, S.K. Sahoo. 2022. Morphology, Length-Weigh relationship, Biology and Conservation Strategies for Least Studied Endemic Catfish, *Rita Chrysea* (Siluriformes: Bagridae) from Mahanadi River System, India. *Journal of Ichthyology* (Accepted). **(IF: 0.603)**

Ferosekhan, S., Giri, A.K., Sahoo, S.K., Radhakrishnan, K., Pillai, B.R., Shankar Giri, S. and Swain, S.K., 2021. Maternal size on reproductive performance, egg and larval quality in the endangered Asian catfish, *Clarias magur*. Aquaculture Research. 00, 1–12. https://doi.org/10.1111/are.15385 **(IF: 2.082)**

Mahapatra, B., Pal, P., Radhakrishan, K., **Ferosekhan, S.,** Sahoo, S.K., 2021. Comparative study on the growth and production characteristics of Asian catfish, *Heteropneustes fossilis* and *Clarias magur* fingerlings. *J. Exp. Zool. India* 24, 665–669.

Ferosekhan, S., Sahoo, S.K., Radhakrishnan, K., Velmurugan, P., Shamna, N., Giri, S.S. and Pillai, B.R., 2020. Influence of rearing tank colour on *Asian catfish*, magur (*Clarias magur*) and pangas (*Pangasius pangasius*) larval growth and survival. *Aquaculture*, 521, 735080. **(IF: 4.242)**

Ahmad, I., Bhat, I.A., Jagtap, D.D., Selvaa Kumar, C., **Ferosekhan, S.** and Afonso, L.O., 2020. New insights of inhibins in ovarian physiology of fish. *Reviews in Fisheries Science & Aquaculture*, 28(2), 247-259. **(IF: 5.893)**

Bharathi,S., Antony C., Rajagopalsamy, C.B.T., Uma, A., Ahilan, B., Lingam, R.S.S., **Ferosekhan, S.,** & Prabhu, E. 2020. Partial replacement of fishmeal with soybean meal and distillers dried grain solubles (DDGS) as alternative protein sources for milkfish, *Chanos chanos* (Forsskal, 1775) fingerlings. *Indian Journal of Fisheries*, 67(4): 62-70. **(IF: 0.50)**

Ferosekhan, S., Sahoo, S. K., Giri, S. S., Das, B. K., Pillai, B. R., & Das, P. C., 2019. Broodstock development, captive breeding and seed production of bagrid catfish, Mahanadi rita, *Rita chrysea* (Day, 1877). *Aquaculture*, 503, 339-346. **(IF: 4.242)**

Sahoo, SK, **Ferosekhan, S**, Giri, SS, Radhakrishnan, K., Panda, D., SriHari, M., Pillai, BR., 2019. Length–weight relationship and growth performance of different life stages of hatchery-produced magur, *Clarias magur* (Hamilton, 1822). *Aquaculture Research,* 50: 1431– 1437. **(IF: 2.082)**

SK Sahoo, **S Ferosekhan**, SS Giri, M Paramanik, K Radhakrishnan., 2018. Reports on common deformities in induced bred *Horabagrus brachysoma* larvae. *Journal of Entomology and Zoology Studies*, 6 (3), 462-465.

d. Peer-reviewed articles submitted to journal (Under review)

Ferosekhan, S., Radhakrishnan, K. and Sahoo, S.K. 2022. Development of larval rearing protocol of endemic catfish Mahanadi rita, *Rita chrysea* for aquaculture species diversification. *Aquaculture* (Under review).

Bairwa, M.K., Swain, S.K., Ail, S.K., **Ferosekhan, S.** and Baruah, A. 2022. Broodstock development, induced breeding and seed rearing of Narayan barb, *Pethia narayani* (Hora, 1937) in captive condition. *Aquaculture* (Under review).

Gokulakrishnan, M., Kumar, R., **Ferosekhan, S.,** Siddaiah, G., Nanda, S., Pillai, B., Swain, S. 2022. Generating circular bio-economy in global aquafeed production by utilizing brewer's spent yeast (BSY) as a sustainable alternate for fishmeal. *Reviews in Aquaculture* (Under review).

Padhi, N., Jena, S.K., Ail, S.K., **Ferosekhan, S.,** Sahoo, S.N., Udit, U.K., Bairwa, M.K. and S.K. Swain. 2022. Effect of tank background color on growth, survival and body pigmentation in filament barb, Dawkinsia filamentosa (Valenciennes, 1844). *Aquaculture Research* (Under review).

Bharathi,S., Antony C., Rajagopalsamy, C.B.T., Uma, A., Ahilan, B., Lingam, R.S.S., **Ferosekhan, S.,** and Prabhu, E. 2022. Complete replacement of fishmeal with soybean meal and distillers dried grain solubles (DDGS) in the diet of milkfish, *Chanos chanos* (Forsskal, 1775) fingerlings. *Indian Journal of Fisheries* (Under Review).

e. Popular articles and booklets

Sahoo, S.K., **Ferosekhan, S.,** Sahoo, S.N., and S.S. Giri. 2021. Some facts on cannibalism *in Wallago attu* and its management during captive seed production. *Aquaculture Asia*. 25(3), July-September 2021. 15-18.

Ferosekhan, S., Sahoo, S.N., Giri, S.S. and Sahoo, S.K. 2021. Scenario of captive production *of Clarias magur* in India. *Aquaculture Asia*, January-March 2021, 25 (1), 10-13.

Sahoo, S.K., **Ferosekhan, S.** and Giri, S.S., 2019. Some facts for the grow-out culture of an endangered catfish, *Clarias magur*. *Aquaculture Asia*, 23, 13-15

Sahoo, S.K., **Ferosekhan, S.,** Giri, S.S., Sivaraman, I., Pillai, B.R. and Swain, S.K. 2021. A booklet on "Magur Farming for Entrepreneurship Development", *ICAR-CIFA Extension Series-63*, pp 10.

Radhakrishnan, K., **Ferosekhan, S.,** Aanand, S., Karthy, A. and Priyadarshani, A. 2018. Advances in Cobia Seed Production and Hatchery Management in India. *World Aquaculture*, 65.

Sahoo, S.K., **Ferosekhan**, S. and Giri. S.S., 2018. Current know how and possibility for grow out culture of an endangered catfish, *Horabagrus brachysoma*. *Aquaculture Asia*, 22 (2), 11-14.

f. Book and book chapter

Book edited

Pillai, B.R., **Ferosekhan, S.,** S.K. Swain. 2021. Advanced Aquaculture Technologies: "System Diversification in Aquaculture". ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India. 70 pp.

Book chapter

Das, P.C. and **S. Ferosekhan.** 2021. Principles and practices of biofloc technology in freshwater aquaculture. Book edited by Pillai, B.R., Ferosekhan, S., S.K. Swain. 2021 on Advanced Aquaculture Technologies: "System Diversification in Aquaculture". ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India. 70 pp.

Sahoo, S.K. and **Ferosekhan, S.** 2018. Seed Production and Culture of *Clarias magur*. In SAARC regional training programme on "Mass breeding and culture technique of catfishes" pp 180. Published by ICAR-CIFA, Bhubaneswar, India and SAARC Agricultural Centre, Dhaka Bangladesh p36-41.

Sahoo, S.K. and **Ferosekhan, S**. 2018. Induced Breeding and Culture of Yellow Catfish, *Horabagrus brachysoma*. In SAARC regional training programme on "Mass breeding and culture technique of catfishes*"* pp 180. Published by ICAR-CIFA, Bhubaneswar, India and SAARC Agricultural Centre, Dhaka Bangladesh p42-46.

Sahoo, S.K. and **Ferosekhan, S.** 2018. Captive Breeding and Culture of *Pangasius pangasius*. In SAARC regional training programme on "Mass breeding and culture technique of catfishes" pp 180. Published by ICAR-CIFA, Bhubaneswar, India and SAARC Agricultural Centre, Dhaka Bangladesh p47-50.

g. Abstract

Pérez García, Lorenzo-Felipe, **Ferosekhan**, Leon-Bernabeu, Izquierdo, Ginés, Afonso, Shin, and M. J. Zamorano. 2021. Influence of estimated breeding value for growth trait on spawning quality in gilthead seabream (*Sparus aurata*) at International Society for Animal Genetics (Virtual), July 26-30, 2021.

Ferosekhan et al., 2019. Broodstock nutritional programming: Influence of broodstock selection (high or low *fads2*) and dietary fatty acid profiles on reproductive

performance and egg quality in gilthead sea bream *Sparus aurata* at **Asian Pacific Aquaculture 2019** during 19-21 July 2019 at Chennai, India.

Ferosekhan et al., 2019. Effect of maternal size on the reproductive performance, egg and larval quality in *Clarias magur*" at **Asian Pacific Aquaculture 2019** during 19-21 July 2019 at Chennai, India.

Ferosekhan et al., 2019. Influence of genetic selection for growth and dietary n-3 LC-PUFA levels on reproductive performance in gilthead sea bream, *Sparus aurata* at **Aquaculture Europe 2019** during 7-10 October 2019 at Berlin, Germany.

Ferosekhan et al., 2018. Influence of dietary fatty acid profile on reproductive performance in gilthead seabream, *Sparus aurata* broodstock selected for high or low *fads2* expression at **International Symposium on Fish Nutrition and Feeding (ISFNF-2018)** during 03-07 June 2018 at Las Palmas de Gran Canaria, Spain.

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.

Turkmen, S., Xu, H., **Ferosekhan, S.,** Zamorano, M. and Izquierdo, M., 2019. Potential role of epigenetics to improve utilization of feeds low in n-3 LC-PUFA in aquaculture at **European Federation of Animal Science 2019-**Conference during 26 to 30 August 2019 in Ghent, Belgium.

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.

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 seabream growth performance, liver fatty acid composition and gene expression. 18th **International Symposium on Fish Nutrition and Feeding, Las Palmas 2018**, Spain.

Subodh Gupta, S., Nuzaiba P. M., **Ferosekhan, S.,** 2018. Nanotechnology: An Emerging Tool for Nutraceuticals and Drug Delivery in Aquafeed at **International Symposium on Fish Nutrition and Feeding (ISFNF- 2018)** during 03-07 June 2018 at Las Palmas de Gran Canaria, Spain.

2. Paper (Oral and poster) presented in national and international conferences/seminar /symposium

1. Presented Oral presentation in the "**Asian Pacific Aquaculture (APA), 2019**" conference organized by the Tamil Nadu Fisheries University, India during 19-21 July 2019 at Chennai, India.

2. Presented Oral presentation in the "**Aquaculture Europe 2019**" conference organized by the European Aquaculture Society (EAS) during 7-10 October 2019 at Berlin, Germany.

3. Presented poster presentation in the **"International Symposium on Fish Nutrition and Feeding (ISFNF), 2018"** organized by the University of Las Palmas de Gran Canaria, Spain during 03-07 June 2018 at Canary Islands, Spain.

4. Presented an oral presentation on **"Breeding and seed rearing of *Pangasianodon hypophthalmus*"** on 17.08.2021, organised by NFDB, Hyderabad, India.
5. Presented an oral presentation on "**Advances in induced breeding and seed production of catfish"** under the five days national webinar of "Advancement in Finfish seed production for SMART Aquapreneurship 13-17 July 2021" on 15.07.2021, organised by College of Fisheries, Agartala, Tripura, India.
6. Presented an oral presentation in the event of National Fish Farmers Day 2021 on "**Prospects of Catfish Farming in Punjab**" on 10.07.2021, organised by Krishi Vigyan Kendra, S.A.S. Nagar (Mohali), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and District Fisheries Department, S.A.S. Nagar, Punjab, India.
7. Presented an oral presentation on "**Magur and Singhi Farming in Northern India**" in the Webinar of "High value Catfish Farming- An emerging profitable venture in Northern India" on 24 February 2021, organized by ICAR-CIFA RRC, Bhatinda, Punjab, India.

3. Conference/seminar/symposiums/webinar/training programme attended

1. Participated in the Webinar on **"Status of the use of Artemia cysts in fish and crustacean hatcheries around the World"** organised by NACA, Bangkok, Thailand on 2 September 2021.
2. Participated in the Webinar on **"Applying radiology and sonography in the aquatic practice"** organised by World Aquatic Veterinary Medical Association (WAVMA), Unit of the University of Veterinary Medicine, Hannover, Germany on 28 July 2021.
3. Participated in the Webinar on **"Digital Aquaculture"** organised by Aquaculture Innovation Centre (AIC), World Aquaculture Society Asian Pacific Chapter (WAS-APC), and Asian Institute of Technology (AIT) on 28 July 2021.
4. Participated in the Webinar on **"Cataloging Omega-3 Science: Introducing GOED's Clinical Study Database"** organised by American Oil Chemists' Society on 25 June 2021.
5. Participated in the Webinar on **"Application of Sensors in Precision Aquaculture"** organised by Swansea University, UK, 25 May 2021.
6. Participated in the Webinar on **"Species Diversification Webinar Series - ASIAN SEABASS FARMING"** organised by U.S. Soybean Export Council during 26-28 April 2021.

7. Participated in the Webinar on **"UK-India Aquaculture Partnerships event"** organised by British High Commission, New Delhi during 8-11 March 2021.
8. Participated in the Webinar on **"Aquafeed Horizons Online: Advances in Processing and Technology & Advances in Ingredients and Formulation"** organised by Aquafeed Horizons Online during 18-19 February 2021.
9. Participated in the Webinar on **"Happy fish: Keys to fish welfare in RAS"** organised by RASTECH, Denmark on 3 February 2021.
10. Participated in the Webinar on **"Infection with Decapod Iridescent Virus 1 (DIV1) and Preparedness"** organised by NACA, Thailand on September 2020.

4. Participation in Training courses

1. Participated in the training programme on "**Data Analytics Using R**" conducted by G.T.N. Arts College, Dindigul, Tamilnadu during 5-9 July 2021.
2. Participated and delivered lecture for "**BiomedAqu Project-Marie Sklodowska Curie Innovative Training Network"** – training on "Rearing of Live Fish for Bone Studies" during 18 - 20 March 2019 at GIA, ULPGC, Spain.
3. Participated 15 days (20 June to 04 July 2018) training course on "**Fish Reproduction and Steroid Hormone quality**" under **PERFORMFISH, EU Project** at Torre de la Sal Aquaculture Institute (IATS), Castellón, Spain.
4. Participated in "**Basic introduction to statistics using R commander**" at University of Las Palmas de Gran Canaria, Spain during 02-04 May, 2018.

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Grupo de Investigación en Acuicultura

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