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NUEVOS RETOS EN SELECCIÓN GENÉTICA PARA LA MEJORA DE LA COMPETITIVIDAD DEL SECTOR ACUÍCOLA

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NEW CHALLENGES IN GENETIC SELECTION TO IMPROVE THE COMPETITIVENESS OF THE AQUACULTURE SECTOR

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“Esta es una historia sencilla, pero no es fácil contarla. Como en una fábula, hay dolor. Y, como una fábula, está llena de maravillas y de felicidad.”

Giosuè Orefice (La vida es bella)

A mis abuelos y mi tía-abuela

Antonio, Paca y Antonia

Sois y seréis siempre mi fuente de inspiración y perseverancia. Os debo mucho

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Index

Figures index

Chapter 0. Summary

1. Resumen

El sector de la acuicultura es un sector relativamente reciente que ha superado en producción a la pesca extractiva. Como consecuencia de la creciente demanda de alimentos para la población mundial, la producción de pescado ha tenido que evolucionar rápidamente y aumentar sus producciones mediante la acuicultura. Por parte de la investigación y de la empresa se han realizado progresos en el ámbito de la reproducción, de la alimentación, del manejo de los lotes, de la prevención de enfermedades y de las instalaciones. Sin embargo, han sido muy escasas las estrategias dirigidas a la mejora genética de las especies acuícolas. Dentro de los programas de mejora genética, el primer criterio de selección se ha centrado en los caracteres de crecimiento. En la actualidad, están empezando a considerarse otros caracteres como la calidad del pescado íntegro y de la carne, y está adquiriendo mayor relevancia la resistencia a enfermedades debido a las grandes pérdidas económicas que ocasiona. En este sentido, la presente tesis doctoral se centra en la propuesta de programas de mejora genética en dorada (*Sparus aurata*) y corvina (*Argyrosomus regius*) que mejoren la competitividad del sector. Por un lado, en el caso de la dorada, que es una especie en la que se ha avanzado más en el estudio de la componente genética para los caracteres de crecimiento, la tesis se centra en la propuesta de selección para caracteres más innovadores como es la resistencia a enfermedades y el perfil de ácidos grasos del filete. Por otro lado, en la corvina se ha comenzado con el estudio de parámetros genéticos para caracteres productivos como el crecimiento o composición del filete ya que al ser una especie emergente se carece de esta información.

El estudio de resistencia a enfermedades en dorada se realizó mediante un reto a juveniles de dorada que fueron inoculados mediante una inyección intraperitoneal con el patógeno *Photobacterium damselae* subsp. *Piscicida*. Previa a la inoculación se realizó una extracción de sangre en la que se determinaron marcadores inmunológicos innatos (nivel de peroxidasa y actividad bactericida) y adquiridos (inmunoglobulinas M). El objetivo del estudio fue determinar si estos marcadores inmunológicos en condiciones nativas podían ser considerados como criterio de selección. Los resultados nos mostraron una correlación genética positiva y alta del peso corporal de los peces frente a la resistencia al patógeno y los días a la muerte, por lo que los peces de mayor peso ofrecían mayor resistencia al patógeno y tardaban más tiempo en morir. Por otro lado, marcadores inmunes como la actividad peroxidasa mostraron heredabilidades medias y una tendencia a una correlación genética positiva con el peso corporal de los peces. Por lo que la actividad peroxidasa se podría incluir en un programa de mejora genética junto con el peso del pez dando lugar a lo que se ha llamado un pez “coping

style", más resistentes no solo a enfermedades sino también a condiciones ambientales, y con mejores caracteres de crecimiento.

El estudio de parámetros genéticos para el perfil de ácidos grasos en el filete de dorada se realizó en una población que fue criada hasta peso comercial en una jaula en el mar Mediterráneo. Dichos individuos fueron muestreados al inicio y al final del engorde para peso corporal y longitud total. Tras el sacrificio de los peces se analizaron diferentes variables de calidad de la carne (proteína, colágeno, grasa y humedad) y el perfil de ácidos grasos del filete. El filete de dorada mostró un elevado porcentaje de ácidos grasos monoinsaturados (MUFA, 44,4%), destacando el 18:1n9c (33,6%), seguido por el porcentaje de ácidos grasos polinsaturados (PUFA, 29,8%), destaca el 22:6n3 (6,8%) y finalmente el porcentaje de ácidos grasos saturados fue 28,3%, siendo el mayoritario el 16:0. Se observó una heredabilidad media para los caracteres de crecimiento y las variables de calidad de la carne, excepto para el colágeno que mostró una heredabilidad baja. Respecto al perfil de ácidos, se observó una heredabilidad media para el ácido graso 18:1n9c, para MUFA y para el ratio n3/n6; y baja para el 22:6n3. Además, se observó una correlación fenotípica negativa entre el contenido en grasa del filete y el porcentaje de PUFA. Por tanto, una selección para incrementar el peso de la dorada acompañada de un incremento del contenido de grasa del filete puede conducir a una disminución del porcentaje de PUFA. El poder incluir el perfil de ácidos grasos del filete como criterio de selección puede ser interesante desde el punto de vista del consumidor por el papel de estos en las enfermedades cardiovasculares y desde el punto de vista del animal por su relación con su sistema inmune.

Para poder llevar a cabo el estudio de parámetros genéticos en la corvina (*Argyrosomus regius*), fue previamente necesario el desarrollo de un test de parentesco mediante un panel de marcadores moleculares microsatélites, ya que no se contaba con él como sucedía en el caso de la dorada. Los paneles de microsatélites son una herramienta robusta y muy eficaz en la realización del pedigrí. El estudio se llevó a cabo con una población de reproductores de corvina y sus descendientes, procedente del grupo Avramar S.L. Inicialmente se probaron 21 marcadores microsatélites, pero tras su ensayo por mala calidad de amplificación, ser poco polimórficos, patrón de bandas poco claro y alelos intermedios nos quedamos con un panel con 8 marcadores microsatélites específicos e interespecíficos de corvina, el cual se denominó Super Multiplex *Argyrosomus regius* (SMAr). Con este panel se mostraron unos resultados de asignación del 95% a una única pareja posible de progenitores.

Finalmente, el estudio en corvina se centró en analizar caracteres de interés comercial, como variables de crecimiento (peso y longitud), caracteres morfológicos (longitud estándar y

altura en el punto medio y en el pedúnculo de la aleta caudal) obtenidos mediante análisis de imágenes y calidad de la carne (contenido de grasa, proteína, humedad y colágeno). Se realizó en la población de corvina procedente de un lote de reproductores de la empresa Alevines del Sureste, del grupo Avramar S.L., que fueron criadas en dos ambientes diferentes (jaula y tanque). Los resultados mostraron que los peces criados en la jaula presentaban mayores valores de caracteres de crecimiento, morfológicos y un mayor porcentaje de grasa, en cuanto al porcentaje de proteína este fue menor que en los peces procedentes del tanque. Además, se obtuvieron heredabilidades medias para caracteres de crecimiento, caracteres morfológicos y porcentaje de grasa, lo que nos indican que son criterios que se pueden incluir en un programa de mejora genética, para obtener individuos mejorados para dichas variables. El análisis de imágenes se mostró de gran utilidad para evaluar variables de crecimiento y morfológicas, nos aporta gran cantidad de información y valores más objetivos, ya que evitamos los errores de las mediciones manuales.

Por los resultados expuestos, podemos concluir que la selección para resistencia a enfermedades va a ser uno de los objetivos de mayor interés en la dorada en concreto, y en las especies acuícolas en general. En otros caracteres de calidad, como el contenido en grasa y su perfil de ácidos grasos, hay que seguir investigando para ver cómo pueden ser implementados en los esquemas de selección. Finalmente, el desarrollo de nuevas especies y tecnologías es otro de los objetivos en acuicultura y sobre ellas se debe comenzar a estudiar en programas de mejora genética.

2. Abstract

The aquaculture sector is a relatively recent sector and which has overtaken extractive fishing in terms of production. As a consequence of the growing demand for food for the world's population, fish production has had to evolve rapidly and increase its production through aquaculture. Research and companies have advanced in the areas of breeding, feeding, herd management, disease prevention, and facilities. However, there have been very few strategies aimed at genetic improvement of aquaculture species. Within genetic improvement programs, the first selection criterion has focused on growth traits. Nowadays, other traits are starting to be considered, such as whole fish and meat quality, and disease resistance is becoming more relevant since it can cause major economic losses. In this sense, this doctoral thesis focuses on the proposal of possible genetic improvement programs in gilthead sea bream (*Sparus aurata*) and meagre (*Argyrosomus regius*) to improve the competitiveness of the sector. On the one hand, in the case of sea bream, a species in which more progress has been made in the study of the genetic component for growth traits, the thesis focuses on the proposal of selecting for more innovative traits such as disease resistance and the fatty acid profile of the fillet. On the other hand, the study of genetic parameters for productive traits such as growth or fillet composition has been initiated in meagre, as it is an emerging species and such information is lacking.

The study of disease resistance in gilthead sea bream was conducted by challenging juvenile gilthead sea bream that were inoculated by intraperitoneal injection with the pathogen *Photobacterium damselae* subsp. *piscicida*. Before inoculation, blood was drawn and innate (peroxidase level and bactericidal activity) and acquired (immunoglobulin M) immune markers were determined. The aim of the study was to determine whether these immunological markers under native conditions could be considered selection criteria. The results showed a positive and high genetic correlation of fish body weight with pathogen resistance and days to death, whereby heavier fish offered greater resistance to the pathogen and took longer to die. On the other hand, immune markers, such as peroxidase activity, showed a medium heritability and a tendency for positive genetic correlation with fish body weight. Therefore, peroxidase activity could be included in a genetic improvement program along with fish weight, resulting in what has been termed a "coping style" fish, more resistant not only to disease but also to environmental conditions, and with better growth traits.

The study of the genetic parameters of the fatty acid profile in sea bream fillet was carried out in a population that was reared to commercial weight in a cage in the Mediterranean

Sea. Samples were taken from those individuals at the beginning and at the end of fattening to determine body weight and total length. After the fish had been slaughtered, different meat quality variables (protein, collagen, fat, and moisture) and the fatty acid profile of the fillet were analyzed. Gilthead sea bream fillet showed a high percentage of monounsaturated fatty acids (MUFA, 44.4%), with 18:1n9c (33.6%) standing out, followed by the percentage of polyunsaturated fatty acids (PUFA, 29.8%), with 22:6n3 (6.8%) standing out, and finally the percentage of saturated fatty acids was 28.3%, with 16:0 being the majority. Medium heritability was observed for growth traits and meat quality variables, except for collagen, which showed low heritability. Regarding the acid profile, medium heritability was observed for 18:1 fatty acid, for MUFA and for ration3/n6; and low for 22:6n3. In addition, a negative phenotypic correlation was observed between fillet content and percentage of PUFA. Therefore, a selection for increased gilthead sea bream weight accompanied by an increase in fillet fat content may lead to a decrease in PUFA. From the consumer's point of view, it may be interesting to include the fatty acids profile of the fillet as a selection criterion because of their role in cardiovascular diseases and from the animal's point of view because of their relationship with its immune system.

To carry out the study of genetic parameters in meagre (*Argyrosomus regius*), it was necessary to previously develop a parentage test using a microsatellite molecular marker panel, since none was available unlike in gilthead sea bream. Microsatellite panels are a robust and very effective tool for parentage testing. The study was conducted with a population of meagre broodstock and their offspring from the Avramar S.L. group. Initially, 21 microsatellite markers were tested, but after testing them for poor amplification quality, low polymorphism, unclear banding pattern and intermediate alleles, we were left with a panel with eight meagre-specific and interspecific microsatellite markers, which was named Super Multiplex *Argyrosomus regius* (SMAr). The results of the 95% assignment to a single pair of possible parents, using the exclusion method implemented in Vitassing software (v.8_2.1), are shown.

Finally, the study in meagre focused on the analysis of characteristics of commercial interest, such as growth variables (weight and length), morphological characteristics (standard length and height at the midpoint and peduncle of the caudal fin) obtained by image analysis, and meat quality (fat, protein, moisture, and collagen content). The study was carried out on a population of 616 meagre from a batch of broodstock from the company Alevines del Sureste, belonging to the Avramar S.L. group, which were reared in two different environments (cage and tank). The results showed that the fish reared in the cage presented higher values of growth and morphological characteristics and a higher percentage of fat, while the percentage of protein

was lower than in the fish in the tank. In addition, mean heritabilities were obtained for growth characteristics, morphological characteristics and fat percentage, indicating that these are criteria that can be included in a genetic improvement program to obtain improved individuals for these variables. Image analysis proved very useful for evaluating growth and morphological variables, providing us with a large amount of information and more objective values, since this avoided possible errors in the case of manual measurements.

From the results presented, we can conclude that selection for disease resistance will be one of the most interesting targets in gilthead sea bream in particular, and in aquaculture species in general. In other quality traits, such as fat content and fatty acid profile, further research is needed to see how these can be implemented in selection schemes. Finally, the development of new species and technologies is another objective in aquaculture that should be studied in breeding programs.

Chapter 1. General introduction

1.0. Aquaculture sector

Aquaculture, according to the Food and Agriculture Organization of the United Nations (FAO 2019), refers to the production of aquatic organisms such as fish, mollusks, crustaceans, and plants. This type of farming requires human intervention to increase the production, use the specific group of fish, feed them, and protect them from predators. Aquaculture is very different depending on where it takes place, from freshwater fish farming in Vietnam to raising shrimp in a salty pond, and cage salmon production off the coasts of Norway and Scotland. Farming also involves individual, or group ownership of the stock being cultivated, feeding planning, location of facilities and management practices in production (FAO 2019). According to the FAO, global aquaculture produced 35.5 million tons in 2000, which increased to 59 million tons in 2010 and to 82.1 million tons in 2018 (Figure 1, (FAO 2019); the world increase in aquatic animal production was around 5.3% yearly for the period 2001-2018. The world of aquaculture was dominated by Asia, with 89% of production in recent decades. The main aquaculture producing countries are Egypt, Chile, India, Indonesia, Vietnam, Bangladesh, and Norway (FAO 2019).

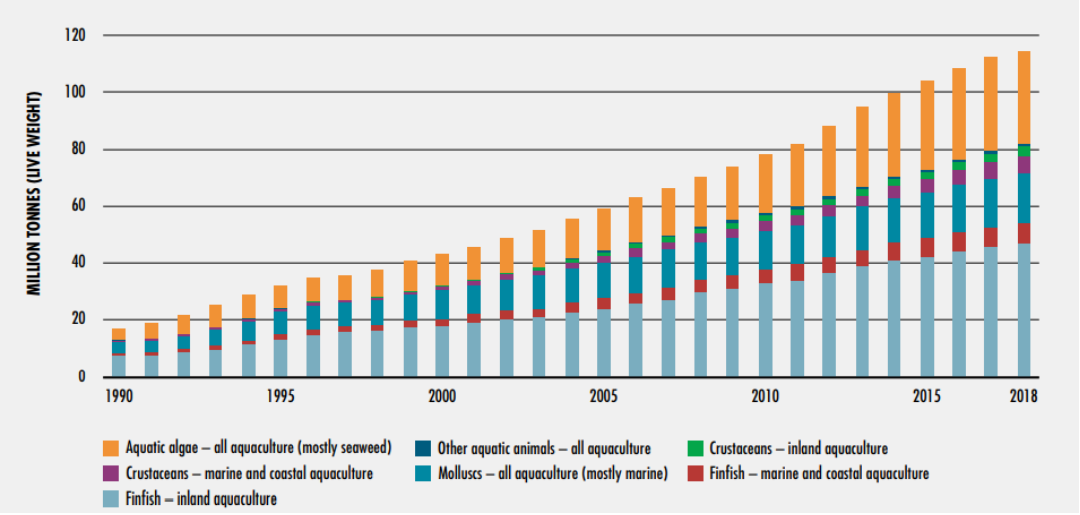

Figure 1. World aquaculture production of aquatic animals and algae, 1990–2018. Source: FAO2020

In the case of the European Union, it has 55,000 km of coastline, the second longest in the world after Canada. It offers environmental and physical climate conditions that are conducive to aquaculture. Moreover, the member states of the European Union are leaders in research, with well-trained human resources (APROMAR, 2020). But, in addition to the demanding standards, the European Union has passed new stringent rules to protect the environment, producing safe food, respecting the environment scrupulously and motivating the

people who work in this area of production (APROMAR, 2020) In 2018, 1,365.12 tons of aquaculture products were harvested. Spain is the member state with the largest aquaculture harvest, with 347.825 tons in 2018, followed by the United Kingdom (197.618 tons) and France (185.650 tons) (APROMAR, 2020). In 2018, 695,886 tons of aquaculture fish were collected in the European Union, where the United Kingdom was the largest producer with 180,476 tons, followed by Greece with 110,220 tons and in third place Spain, with 60,535 tons (Figure 2) (APROMAR, 2020).

Based on Spanish and Mediterranean aquaculture production, two species of interest were selected for these studies, thus, gilthead sea bream and meagre were studied.

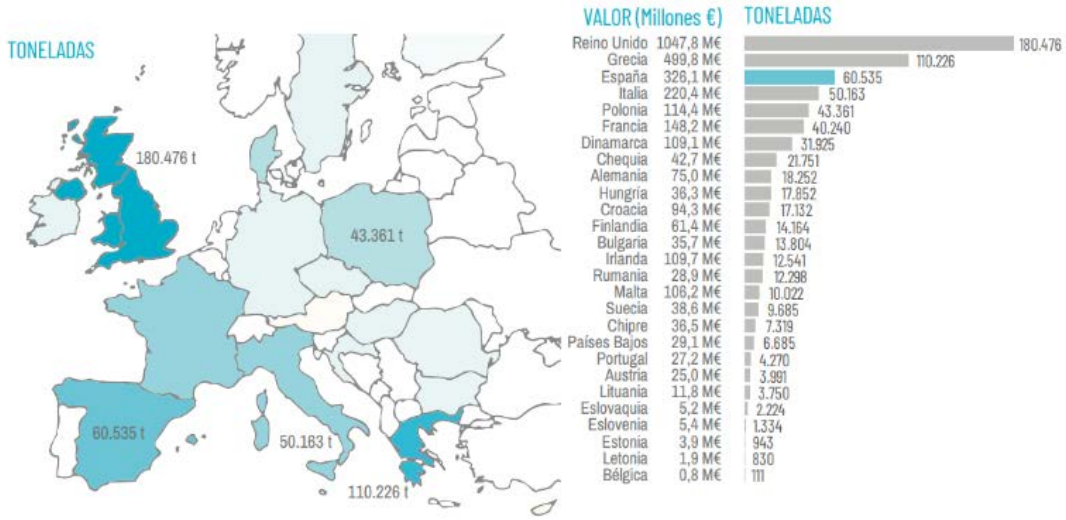

Figure 2. Distribution of aquaculture finfish production in the member states of the European Union by volume (tonnes) and value (millions of Euros) in 2018 (FAO).

1.0.1 Gilthead sea bream (Sparus aurata L.)

The gilthead sea bream is a marine teleost fish belonging to the Sparidae family and the Sparus genus that can be found from 30 to 50 m depth (Figure 3), in the Mediterranean and the Black Sea, and in the Eastern Atlantic, from the British Isles, Strait of Gibraltar to Cape Verde and around the Canary Islands (Aurelio, 2008). It may also be found in marine and brackish-water environments like coastal lagoon and estuarine areas (FAO 2019), due to its euryhaline and eurythermal habits, growing very fast when the temperature is 25-26ºC and ceasing to feed if the temperature falls to 12-13ºC (Aurelio, 2008). It is one of the most important species in the Mediterranean area for fisheries and aquaculture, with a production of 254.406 tons in 2019 through aquaculture in Europe and the rest of the Mediterranean countries. The main producers of gilthead sea bream in 2019 were Turkey, Greece, Egypt, Tunisia, and Spain, in that order. Spain reached 13.521 tons in 2019, which represents 5.4% of European production (APROMAR, 2020).

Figure 3. Specimen of gilthead sea bream. Source: own edition

Consequently, gilthead sea bream has a great interest to the industry and the aquaculture sector; for this reason, these sectors seek to be more competitive and demand further improvements in production, such as better quality and lower production costs. Gilthead sea bream is protandrous hermaphrodite, in the first cycle 100% of the population are males and this changes at the end of the second reproductive cycle. This sex change is initiated by some individuals, but at the beginning of the third reproductive cycle, 80% mature as females and 20% develop testes again (Chaves-Pozo et al., 2009, 2005; Liarte et al., 2007), this avoids knowing the genealogy of fish (breeders and hatchlings). Fish reproduction occurs through

mass spawning, i.e., there is a batch of broodstock (males and females) in the same tank. Eggs are collected from the tank and the genealogy of the fish is unknown (broodstock and fingerlings), and pedigree reconstruction is essential in order to estimate genetic parameters (Cárdenas, 2010).

1.0.2. Meagre (Argyrosomus regius)

Meagre (*Argyrosomus regius*) has been one of the most important fish in Mediterranean aquaculture diversification. Meagre is a teleost fish of the Sciaenidae family (Figure 4); it can be found from 15 to 200 m depth, on the Atlantic coast of Europe, of the Mediterranean Sea and Black Sea and on the east coast of Africa (Cárdenas, 2010), and it may also be found in estuaries and coastal lakes (Griffiths and Heemstra, 1995). Meagre has been important in the aquaculture system as a potential of diversification of European aquaculture, due to its fast growth (around 800-1000 grams in 18 months) with good water temperature conditions, and low food conversion ratio (1.74) (Cárdenas, 2010). Annual production in the Mediterranean area exceeds 41.295 tons, with the main producing countries being Egypt, Spain (which produced 3.650 tons in 2019), Turkey and Greece, in that order. (APROMAR, 2020) This species is easy to adapt to captivity, since it is characterized by a tolerance to a wide range of salinity (5-39 ‰) as well as temperature (13-28ºC) (Cárdenas, 2010).

Figure 4. Specimen of Meagre. Source: own edition

To the consumer, meagre has an attractive body shape as a whole fish commodity, low fat fillet content and good processing yield. In Spain, the professionals in fish sales believe that "the consumption of fillet of fish will continue to increase in the next years", due to a new lifestyle habitat young ages, or because of other reasons such as people cooking less, and preferring to consume prepared products (La et al., 2006). With regard to the quality of the fish,

the external appearance is the most important characteristic, since when there is any damage or malformation it is usually irreversible in terms of the morphological aspect, and this in turn produce a decrease in the quality of the final product. In this case, some deformities have been described in meagre, such as liver granulomas (Carvalho et al., 2019) that can lead to reduced growth performance and thus fish which are visually unacceptable for the market. Poli et al. (2003), Piccolo et al. (2008) and Fountoulaki et al. (2017) revealed meagre to be a very different species from other aquaculture ones, highlighting its high growth rate, low food conversion ratio and its low-fat content, both mesenteric and muscular (Figure 5). We must not forget the quality of the fatty acid profile of fish, as it is one of the main sources of omega 3 and 6 fatty acids that vertebrates are not able to synthesize and need to incorporate into their diet, in this case through fish consumption (Calder, 2006). For these reasons, the meagre is an emerging species with great potential, where the field of improvement is still very wide in all areas, including: growth characters, morphological characters, disease resistance, final product quality, processing yield, batch management, and feed intake efficiency (Janssen et al., 2017).

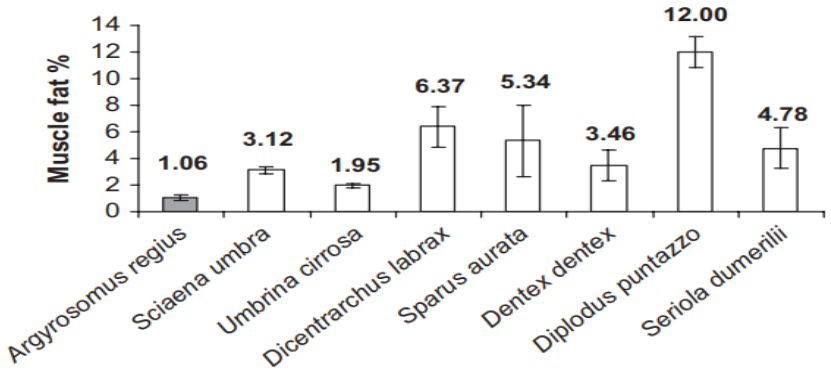

Figure5. Muscle fat contents of meagre in comparison with other reared Mediterranean fish species. Values are average values, and bars represent standard deviations. Source: (Grigorakis et al., 2011).

1.1. Immune system of fish

The immune system is defined as a system capable of protecting the body against agents that can cause disease or infection (Flajnik and Du Pasquier, 2008). The fish immune system relies on both adaptive and acquired immune responses (Rubio-Godoy, 2010). The innate immune system is characterized by the protection of the individual without any type of exposure

to the pathogen, since it acts as the first line of defence against a foreign agent, until the activation of the specific system (Figure 6). The specific system is activated by the response of an invading pathogen and after repeated exposure (Rauta et al., 2012).

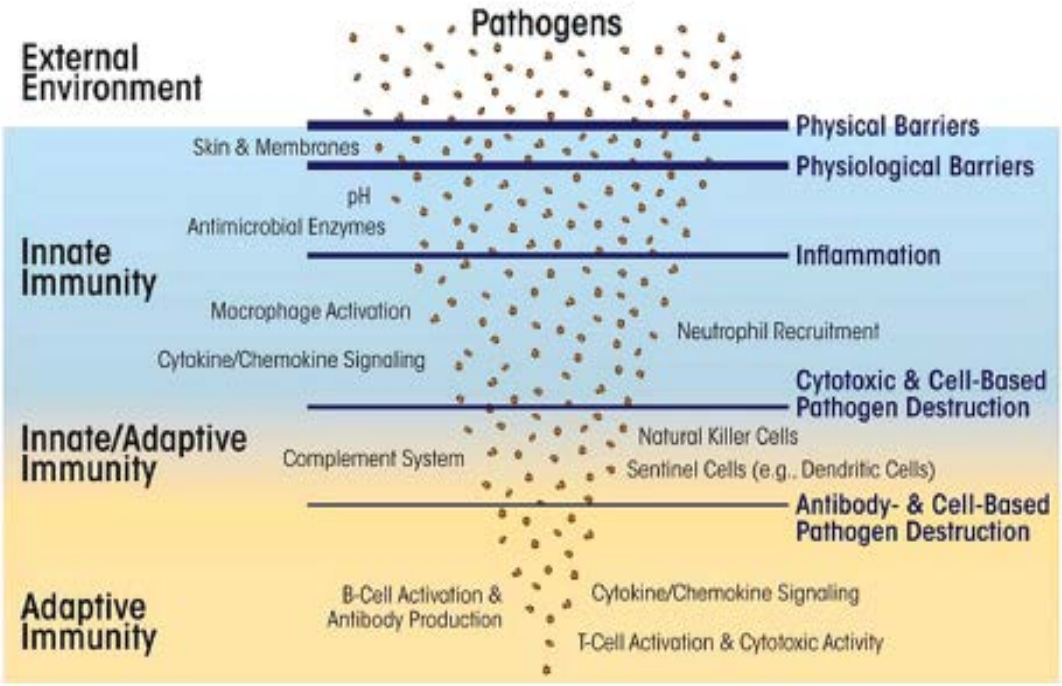

Figure 6. Immune system in fish (Spiering, 2015).

The innate immune system is of great importance in fish, since it is the first to send the signal to protect the individual from infection and connects with a great variety of mechanisms which seems to be more important in fish than in higher vertebrates (MacKenzie et al., 2004). It consists of physical barriers (scales, skin, gills, and mucosal barriers in general), humoral factors (receptors associated with cells and soluble molecules of plasma and other body fluids, such as the complement system, natural antibodies, NAbs, cytokines/chemokines) and cells (phagocytic cells, nonspecific cytotoxic cells, epithelial and dendritic cells).

Fish are constantly in contact with possible pathogens or other harmful agents, owing to them being submerged in an aquatic environment. Therefore, the physical defence barriers play an important role, and the scales, the epithelial cells that line the skin, the gills, the digestive tract, and the mucus that surrounds them are the first defence barrier of the innate immune system (Magnadóttir, 2006, 2010). The mucus that surrounds it contains a wide range of immune substances, such as lectins, lysozyme, peroxidase, antibacterial and IgM, among others, which inhibit the entry or even the elimination of pathogens (Valenzuela et al., 2017).

The cellular response is defined by the physical barrier and the specialized cells, which are capable of killing and digesting pathogens if the latter break down the physical barriers (Aoki et al., 2008). Cells of the innate immune system of teleost fish are phagocytic cells, which recognize and eliminate invading pathogens and other molecules from damaged tissues (Magnadottir, 2010). The humoral response in teleost fish is composed of many non-specific defence substances, such as growth inhibitors, various lytic enzymes, and components of complement pathways, mainly lectins, natural antibodies, cytokines, chemokines, and antibacterial peptides (Magnadóttir, 2006). Peroxidase is known to be one of the most important enzymes, it uses hydrogen peroxide and produces ions to form chlorides and chloramines; these are very toxic microbicidal agents and are important in an immune defence pathway known as the respiratory burst (Whyte, 2007). Peroxidase, which is found in serum, but also in mucus, is considered essential for mucosal immunity and skin defence (Guardiola et al., 2014). Proteases, which are often known to be a group responsible for the degradation of pathogens or other substances, also act by activating and increasing the production of some other immunological components, such as immunoglobulins and antimicrobial peptides (Cho et al., 2002). Teleost fish have three different isotopes of immunoglobulins (Ig) IgM, IgT and IgD (Piazzon et al., 2016). Teleost's produce an Ig that resembles mammalian IgM, present in the membrane and in soluble form. On the one hand, it is secreted by B cells when it appears in soluble form and appears in blood and other fluids, playing an immunological role (Cuesta et al., 2004). IgT, which is found in mucous membranes, is thought to have a similar function to mammalian IgA (Du et al., 2016).

1.2. Disease resistance

With regard to disease resistance, selective breeding programs play a key role in hindering the spread of pathogens, to achieve long-term control of the disease (Das and Sahoo, 2014) and because genetic improvement is cumulative and permanent (Doan et al., 2017). Today, many pathogens affect different populations of fish, such as *Photobacterium damselae* subspecies *piscicida* which has a greater impact on sea bass and gilthead sea bream. The main pathogenic microorganisms isolated affecting gilthead sea bream production are *Vibrio* (67.8%), *Pseudomonas* (13.5%), *Photobacterium damselae* subsp. *piscicida* (6.7%), *Cytophaga/Flexibacter-like bacteria* (4.8%), *Aeromonas* (0.5%), and *Gram-positive bacteria* (6.7%). Although the highest percentages of isolates corresponded to *Vibrio* and *Pseudomonas spp*., the strains of *P. damsela* subsp. *piscicida* caused epizootics with the highest degree of

mortalities, thereby resulting in severe losses for the fish farming industry (Balebona et al., 1998). Although that disease was first described in wild populations of white perch and striped bass, the natural hosts of the pathogen currently include a wide variety of marine fish. That pathogen is the cause of pasteurellosis, which is a bacterial sepsis provoked by halophilic bacteria. The observable symptoms are nodules of between 0.3-0.5 mm in some organs, externally the distended stomach is visible and internally a congestion and dilatation of the digestive tract (Figure 7) (Magaririos et al., 1997).

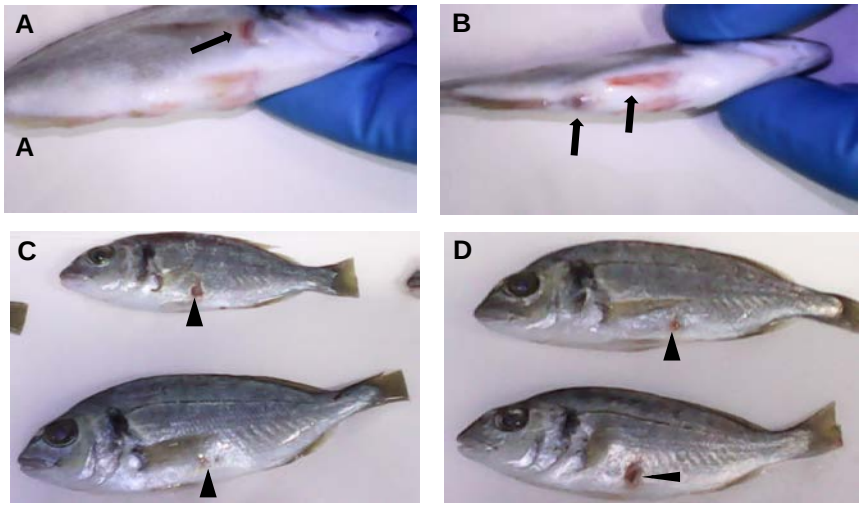

Figure 7. Representative images of the haemorrhage around the bases of the fins (A) and the urinogenital opening (B) and along the pelvic fins (B) and the skin lesions of several sizes (C, D) observed as clinical signs in gilthead seabream specimens intraperitoneally injected with $8x10^4$ cfu of Phdp/fish. Black arrows: haemorrhages, black arrow heads: skin lesion. source: own edition

Disease resistance can be defined as the ability to resist and control an infection, since all animals are susceptible to the initial infection, but differ in their ability to hinder the entry of pathogens, their replication, release, and survival (Dekker et al., 2016). Challenge tests are the most used to investigate if a fish has resistance, whereby fish are subjected to a controlled infection in a standardized environment. These characteristics minimize variations due to uncontrolled sources, maximize the reproducibility of the procedure, and improve the interpretation of data in terms of individual tolerance to the pathogen. For sanitary reasons, infected fish cannot be chosen as future breeders.

1.3. Selective breeding programs in fish

1.3.1. Molecular markers

The most common technologies used in DNA markers for application in aquaculture have been allozymes (Reilly et al., 1999); restriction fragment length polymorphism (Eric S. Lander and David Botst, 1989); random amplified polymorphism (Huang et al., 2000); amplified fragment length polymorphism (Li and Guo, 2004; Vos et al., 1995); microsatellites (Lee-Montero et al., 2013); nuclear and mitochondrial DNA sequencing (Reilly et al., 1999); and single nucleotide polymorphisms (SNPs) (Peñaloza et al., 2021). Currently, microsatellites and SNPs are the most common markers.

✓ Microsatellites

Microsatellites are simple sequences that change in size from one to six base pairs (bp) and are known as tandemly repeated nucleotide motifs and are between 20 and 100 bp in length (Li et al., 2021). With a high mutation rate and genome-wide distribution, microsatellites can have a major impact on the genome through many mechanisms (Adams et al., 2016). This technique has the advantage of offering a high, dominant and highly reproducible morphic information content, which is easily and accurately scored on all DNA markers.

✓ Single Nucleotide Polymorphisms (SNPs)

SNPs are one of the most numerous polymorphisms in the genome and consist of a DNA sequence variation that occurs when a single nucleotide in the genome is different at a particular site. SNPs can occur in both coding and non-coding DNA. The disadvantage of SNPs is that they have less information than microsatellites: five SNPs provide the same information as one microsatellite (Gjedrem, T. and Baranski, 2009). This disadvantage its compensated for by the development of high-throughput SNP technology, such as that produced by Illumina™ and Affymetrix™. More than a million SNPs can be genotyped in a single trial, and the price has dropped in recent years

The use of molecular markers such as microsatellites allows pedigree reconstructions, thereby enabling the detection and avoidance of inbreeding. Concurrently, the development of multiplex microsatellites PCRs allows geneticists to reduce the economic cost per reaction. Several multiplex PCRs have been described for gilthead sea bream by Launey et al. (2003) and Brown et al. (2005); and more recently by Navarro et al. (2008), Porta et al. (2010) and Borrell et al. (2011). In the family of the Scianids, many microsatellites have been described:

30 microsatellites have been described in the red drum (Sciaenops ocellatus) by Saillant et al. (2004) and 30 others by Turner et al. (1998). In yellow meagre (*Pseudosciaena crocea*) 22 microsatellites were described by Li et al. (2008) and 11 by Chang et al. (2009). In Acoupa Weakfish (*Cynosion ocupa*), 17 were described by Farias et al. (2006); in Japanese meagre (*Argyrosomus japonicus*), 15 microsatellites were described by Archangi et al. (2009). In the case of meagre (*Argyrosomus regius*) only a few studies (Nousias et al., 2020; Soula et al., 2012) showed the multiplex they used, additionally we can find 23 specific microsatellite markers in the NCBI database (Porta et al., 2010).

The use of molecular markers such as microsatellites enables not only pedigree reconstructions, but also the detection of inbreeding problems. The use of microsatellite markers helps us to evaluate the genetic variability within a population and between different populations, which is of great help in distinguishing between populations that are very similar genetically (Segvic-Bubic et al., 2011). Genetic variability is considered an important trait for animal species that have undergone domestication processes, since those that present higher levels of variation will consequently have a high additive genetic variance for the different productive traits (Alarcón et al., 2004). Brown et al. (2005) showed the contribution of the reproducers and the offspring in populations of gilthead sea bream; they found a high variance in the size of the family and many reproductive non-contributions broodstock, with a remarkably reduced number of population size (Ne). Ne is a value that indicates the frequency with which genes are expressed generation after generation; it should be noted that a large Ne is related to smaller changes. Although Ne is mainly related to the size of the batch of broodstock involved in the creation of the next generation, it is always smaller than the observed number of broodstock. Factors such as the effective size of the breeding population, unequal parental participation (males and females) and selection influence parental participation in the next generation. Variables such as effective Ne and genetic drift, both closely related, arise from the phenomenon of inbreeding, where Ne is equal to $1/(2\Delta F)$), and ΔF is the inbreeding rate per generation (D.S.Falconer, 1981).

At the same time, the development of multiplex PCRs has helped geneticists to reduce the economic cost per PCR reaction. This is a cheap and more widespread method, which has become very extended and developed in the reproduction of certain aquaculture species. Several studies have already described different multiplex PCRs for gilthead sea bream (Brown et al., 2005; Launey et al., 2003), with new ones still being developed (Borrell et al., 2011; Navarro et al., 2008; Porta et al., 2010). In our study we used the first reproducible, uniform and standardized gilthead sea bream panel (SMsa1; Super Multiplex Sparus aurata) which consists

of eleven high-quality and variability specific markers (Lee-Montero et al., 2013), and a standardized meagre panel (SMAr; Super Multiplex *Argyrosomus regius*) is proposed.

1.3.2. Breeding programs

Breeding programs are tools that should be applied in all production systems. The goal of the genetic program is to determine which animals would most efficiently use resources and grow best in captive conditions. The history of animal breeding began centuries ago, before the elaboration of its theoretical bases, with great success in those years. It is not known exactly when artificial selection began, but we do know that it played an important role in the domestication of many animal species; in this case we can say that artificial and natural (unintentional) selection played a relevant role in past times (Gjedrem, 2005). Modern breeding theory is very recent, its use began early in the last century. The principle of transmission from one generation to the next is the basis for the Mendeley genetics. This theory was first published by the Austrian monk Gregor Johann Mendel in 1866, he began working with plants and discovered the particulate law of inheritance (Gjedrem, 2005). Later, in 1908, Hardy-Weinberg provided the basic information for the theory of population genetics, which was called the study of Mendelian genetics in populations. This study is limited to the development of the calculation of the inheritance of qualitative traits, which can be influenced by one or two genes, and these experimental results are expressed in frequencies of genotypes and phenotypes.

The first effective domestic animal breeding programs were developed in the 1930s in chickens, dairy cows, and pigs, after the development of quantitative genetics. Since then, improvements in production have been remarkable (Figure 8). In the case of aquaculture systems, the application of quantitative genetics has been limited, but fish farming started in China around 5000 years ago with pond culture of carp production, and some growth and disease resistance experiments were carried out in the 1920s (Gjedrem, 2005).

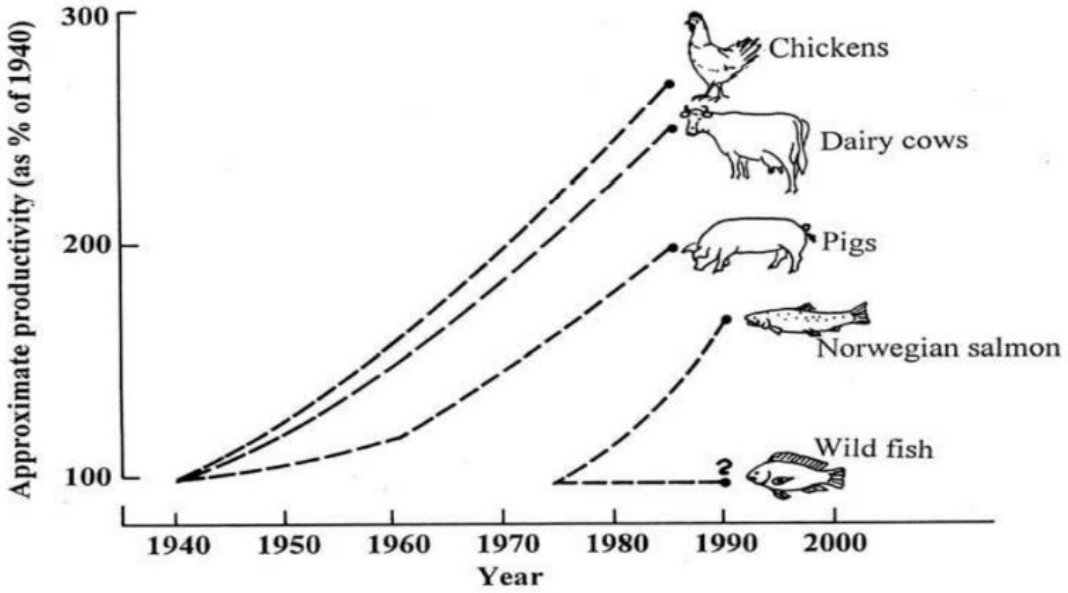

Figure 8. Evolution of genetic research from the 1940s to the present in mammals and domestic birds. by Gjedrem 2005.

Nowadays, due to the late development of breeding programs in fish, information about the genetic component for different traits is scarce for most cultivated species. Breeding programs are based on the estimated genetic parameters of characters on the heritability and genetics correlations between traits. Heritability is a measure of relativity to a proportion of variability genetics versus to an environment factor, such as the cultivation area or the geographical area. The knowledge of the relationship between individuals is necessary to estimate heritability.

In aquaculture, the implementation of a selection scheme usually starts with the optimization of different growth traits such as weight, length, and growth rate, in order to obtain a marketable product in an optimal time, with the consequent lower consumption of feed and improvement in the conversion rate. The first estimation of genetic parameters started in the 1970s, Aulstad et al. (1972) published some of the first heritability estimates of aquaculture in rainbow trout culture for body weight and length and Kirpichnikov (1972) in common carp fingerlings for body weight. Today, breeding programs must focus on documenting the genetic gains achieved and the increased monetary value of the results obtained, in order to be able to carry out a large breeding program under industrial conditions (Gjedrem, T. and Baranski, 2009).

The economic interest that was confirmed in the production of Atlantic salmon and rainbow trout has shown a response of 10 ± 15% growth rate per generation, in this case only the growth rate and body weight were included (Gjedrem, 2000). Until recently, the estimation of genetic parameters was limited only to growth traits. However, with the change in the consumer profile, the selection strategy includes other traits such as knowing the composition of the meat and good processing yield.

In recent years, genetic improvement work has focused on genetic parameter estimates for growth traits in meagre (Nousias et al., 2020), Atlantic salmon (Powell et al., 2008), rainbow trout (Kause et al., 2007) and common carp (Kocour et al., 2007); for growth and carcass yield traits in gilthead sea bream and tilapia (García-Celdrán et al., 2015; Nguyen et al., 2010), growth and energy metabolism estimates in gilthead sea bream (Perera et al., 2021), to mention just a few of the most outstanding works. Similarly, there have been many studies that focus on growth and feed efficiency traits, so there is still a long way to go in genetic improvement programs in aquaculture species. Later, other traits related to the processing yield were included in other fish breeding programs, because of the economic interest of these traits when fish are processed (García-Celdrán et al., 2015; Navarro et al., 2009a). Furthermore, the fresh appearance of the fish is very important and linked to the morphology and nutritional value. In recent years, the importance of fish as a high-quality source of nutrients for the human diet has strengthened (Mccullough et al., 2002), the customer's main concern about aquaculture products is their quality, i.e., their safety, freshness, and health value (Matos et al., 2010). Fatty acids play an important role in the immune and inflammatory process; some fatty acids, such as polyunsaturated fatty acids (PUFA), are considered immune cells that are associated with non-specific immune disorder, specific immune and immune-related gene expression (Chen et al., 2016).

1.3.3. New technologies in breeding programs

It seems that more and more studies are incorporating technological tools for inclusion in breeding programs (Costa et al., 2013a; Elalfy et al., 2021; Navarro et al., 2016), which can be classified into invasive and non-invasive technologies. In recent studies, it has been decided to include some of the non-invasive technologies (NiT) in different aquaculture species (Bosworth et al., 2001; Costa et al., 2013b; Grassi et al., 2018; Halim et al., 2013). One such techniques is the Distell Fish Fat Meter device ("Distell.com", figure 9), which is used to determine the percentage of lipids in the muscle as a non-invasive measure, using a microstrip sensor capable of determining the percentage of fat in the muscle, thanks to the water content. On the other hand, for morphological characters Navarro et al., (2016) developed a software (IMAFISH_ML), which through image analysis provides us with 27 measurements of different morphometric traits of fish, in three commercial fish species: gilthead sea bream *(Sparus aurata* L.), meagre (*Argyrosomus regius*) and red porgy (*Pagrus pagrus*). On the other hand, automatic analysis based on invasive technology such as Infrared Spectroscopy (NIR), using the FOODSCAN LAB equipment (FOSS, Denmark) is used to determine the chemical composition of the meat. Thanks to these techniques, it is possible to measure body composition, such as protein, fat, moisture, and collagen.

Figure 9. Fish Fat Meter device. Source: Distell.com

Elalfy et al. (2021) observed in their study that the fat content analyzed by both methodologies Fish Fat Meter and NIR reported 9.47% and 6.37% respectively, concluding that measurements made with non-invasive techniques were slightly higher than those made by invasive techniques, and pointing out that there is a high and positive correlation between both variables with respect to growth traits, assuming that the non-invasive technique is faster, more convenient, easier and cheaper to evaluate. In addition, we cannot forget that the incorporation of these non-invasive technologies in breeding programs helps us to select fish for quality traits, such as fat, in the individuals themselves, reducing the time and cost involved in selecting the next generation of individuals.

In addition to molecular markers to know the relationship between individuals and the different techniques to evaluate the different characters of fish, it is necessary to use tagging to identify individuals throughout their lives to follow a process of individual traceability. The traceability of the individual is carried out thanks to the individual tagging of the fish with a physical system such as the Passive Integrated Transponder (PIT). The PIT has the advantage of being easy to handle, automatable, with a wide range of numerical codes, and offering great security when used on fish, with great confidence for use on small fish (Navarro et al., 2006). For this reason, the PIT needs to be tested in different species because of their unequal susceptibility to anesthesia and manipulation, capacity of recovery, growth rate, and morphology. Thus, different study has been carried out in Atlantic salmon, (*salmo salar*) (Gries and Letcher, 2002); chinook salmon (*Oncorhynchus tshawytscha*) (Dare, 2003); Eurasian perch (*Perca fluviatilis*) (Baras et al., 2000) and bullhead (*Cottus gobio*) (Bruyndoncx et al., 2002).

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Chapter 2. Objectives

El objetivo general de esta tesis doctoral es investigar algunos de los retos actuales de la acuicultura y estudiar cómo se pueden incluir en los programas de mejora genética. Para ello, se propone el estudio de los componentes fenotípicos y genéticos de los siguientes retos específicos:

- **Objetivo 1**. Resistencia a enfermedades. Caso de estudio de resistencia al patógeno *Photobacterium damselae* subsp. *piscicida* de juveniles de dorada (*Sparus aurata* L.).

- **Objetivo 2.** Calidad de la carne y perfil de ácidos grasos. Caso de estudio, la dorada a tamaño comercial (S*parus aurata* L.).

- **Objetivo 3.** Estandarización de paneles de marcadores microsatélites en corvina. Caso de estudio, desarrollo del primer panel en corvina (*Argyrosomus regius*).

- **Objetivo 4.** Desarrollo de nuevas especies y tecnologías. Caso de estudio, desarrollo de la corvina (*Argyrosomus regius*) para caracteres de crecimiento, caracteres morfológicos mediante análisis de imágenes y calidad de la carne.

Para llevar a cabo estos objetivos, la tesis doctoral se ha dividido en cuatro capítulos (Figura 10), dos en dorada (artículo 1 y 2) y dos estudios en corvina (artículo 3 y 4).

The general objective of this doctoral thesis is to investigate some of the current challenges in aquaculture and to study how they can be included in genetic improvement programs. For this purpose, the study of the phenotypic and genetic components of the following specific challenges is proposed:

- **Objective 1**. Disease resistance. Case study on resistance to the pathogen Photobacterium damselae subsp. piscicida of juvenile gilthead sea bream (*Sparus aurata* L.).
- **Objective 2**. Meat quality and fatty acid profile. Case study, commercial-sized gilthead sea bream (*Sparus aurata* L.).
- **Objective 3**. Standardization of microsatellite marker panels in meagre. Case study, development of the first panel in meagre (*Argyrosomus regius*).
- **Objective 4**. Development of new species and technologies. Case study, development of meagre (*Argyrosomus regius*) for growth characters, morphological characters by image analysis and meat quality.

To achieve these objectives, the doctoral thesis has been divided into four chapters (Figure 10), two on gilthead sea bream (paper 1 and 2) and two studies on meagre (paper 3 and 4).

Gilthead Seabream (*Sparus aurata*)	**OBJECTIVE 1.** *Paper 1*. Genetic Parameters for *Photobacterium damselae* subsp. *piscicida* Resistance, Immunological Markers and Body Weight in Gilthead Seabream (*Sparus aurata*). **OBJECTIVE 2** *Paper 2*. Genetic Analysis of the Fatty Acid Profile in Gilthead Seabream (*Sparus aurata*)
Meagre (Argyrosomus regius)	**OBJECTIVE 3** *Paper 3*. Development of the fisrt microsatellite multiplex PCR panel for meagre (*Argyrosomus regius*), a commercial aquaculture species **OBJECTIVE 4** *Paper 4*. Phenotypic and genetic components for growth, morphology and flesh quality traits of meagre (*Argyrosomus regius*) reared in tank and sea cage with different stock density.

Figure 10. Informative scheme of the connection of the objectives with the papers of the thesis.

Chapter 3. Compendium of papers

1.0. Abstract paper 1:

Aquaculture is a relatively recent sector that has surpassed extractive fishing in terms of production. However, genetic improvement strategies for aquaculture species have been very scarce and, within genetic improvement programs, the first selection criterion has focused on growth traits. Now other traits, such as whole fish and meat quality, are beginning to be taken into account, and disease resistance is becoming more important, as it can cause significant economic losses. The study of disease resistance in gilthead sea bream was conducted by challenging juvenile gilthead sea bream, at 272 days post-hatching (dph), that were inoculated by intraperitoneal injection with the pathogen Photobacterium damselae subsp. piscicida and weighed. Before inoculation, blood was drawn and innate (peroxidase level and bactericidal activity) and acquired (immunoglobulin M) immune markers were determined. From this time, survival fish were recorded for 9 days, and days to death was registered. The aim of the study was to determine whether these immunological markers under native conditions could be considered selection criteria. The results showed a positive and high genetic correlation of fish body weight with pathogen resistance and days to death, whereby heavier fish offered greater resistance to the pathogen and took longer to die. On the other hand, immune markers, such as peroxidase activity, showed a medium heritability and a tendency for positive genetic correlation with fish body weight. Therefore, peroxidase activity could be included in a genetic improvement program along with fish weight, resulting in what has been termed a “coping style” fish, more resistant not only to disease but also to environmental conditions, and with better growth traits.

Paper 1. Genetic Parameters for *Photobacterium damselae* subsp. *piscicida* Resistance, Immunological Markers and Body Weight in Gilthead Seabream (*Sparus aurata*)

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Abstract

A challenge test for *Photobacterium damselae* subsp. *piscicida* (*Phdp*) resistance was carried out in two juvenile populations of gilthead seabream (*Sparus aurata* L.): F2_ATL and F0_MED. At 250 days post-hatching (dph), a fish plasma sample was collected to measure humoral immune markers (peroxidase activity, bactericidal activity, and IgM immunoglobulin levels), and at 272 dph fish were weighed and inoculated with bacteria *Phdp*. From that time onwards, surviving fish were recorded for nine days, and days to death was registered. Heritabilities for body weight and *Phdp survival* were moderate, although for days to death the heritability was low. Regarding humoral immune markers, for peroxidase activity it was moderate, and for IgM levels and for bactericidal activity it was low. Genetic correlations for body weight with *Phdp* survival and days to death were high and positive, while with peroxidase activity and IgM levels they tended to be positive, although these estimates were not accurate. Regarding genetic correlations between *Phdp* survival and humoral immune markers, they were very high, positive with peroxidase activity, and negative with IgM levels and bactericidal activity. Some humoral immune markers, particularly peroxidase activity, along with performance traits such as body weight and absence of deformities, are proposed to be included in a selective breeding program to raise fish that are capable of coping with diseases.

Keywords: Gilthead seabream (*Sparus aurata*), *Photobacterium damselae* subspecies *piscicida*, Immunoglobulin M, Peroxidase activity, Heritability.

1.1. Introduction

The gilthead seabream is one of the most valuable species in the Mediterranean basin both for fisheries and aquaculture. Total production in Mediterranean countries reached 253,000 metric tons in 2019. The three most important countries producing gilthead seabream were Greece, Turkey and Spain, in that order (APROMAR, 2020). As production increases, so disease outbreaks have become serious threats causing important economic losses and a high environmental impact. The farming activity and the open design of Mediterranean aquaculture systems facilitate the transmission of infectious pathogens within and among farm facilities (Arechavala-Lopez et al., 2013). The main pathogenic microorganisms isolated affecting sea bream production are *Vibrio* (67.8%), *Pseudomonas* (13.5%), *Photobacterium damselae* subsp. *piscicida* (6.7%), *Cytophaga/Flexibacter-like bacteria* (4.8%), *Aeromonas* (0.5%), and *Gram-positive bacteria* (6.7%). Although the highest percentages of isolates corresponded to *Vibrio*

and *Pseudomonas spp*., the strains of *P. damsela* subsp. *piscicida* caused epizootics with the highest degree of mortalities, thereby resulting in severe losses for the fish farming industry (Balebona et al., 1998).

Photobacterium damselae subspecies *piscicida* (*Phdp*) was first described in wild populations of white perch and striped bass, but the natural hosts of the pathogen currently include a wide variety of marine fish. *Phdp* causes "pseudotuberculosis", which is recognized by granulomatous-like lesions in the internal viscera, particularly in the spleen and kidney. External symptoms are weight loss, a darkening of the skin, localized necrosis in the gills and skin, distended stomach, and external hemorrhages (Magariños et al., 1996). Internal symptoms are nodules of between 0.3-0.5 mm in some organs and a congestion and dilatation of the digestive tract (Magariños et al., 1996). The disease develops rapidly into an acute septicemia condition characterized by conspicuous splenomegaly, and high mortalities have been observed in gilthead seabream from Atlantic and Mediterranean areas. Transmission of the pathogenic bacteria can be vertical, through gonadal fluids, as well as horizontal through the water route, this latter route enables the bacteria to infect its host through the gills, the digestive system, and the skin (Borrego et al., 2017). The proliferation peak of said pathogen occurs with the high temperatures of summer (Magariños et al., 2001). Common interventions to prevent or control the disease are vaccines and antibiotics. Several vaccines against *Phdp* have been developed and applied, however, they are not always effective enough, and the vaccination protocol involves several bath and oral exposures (Andreoni and Magnani, 2014). Antibiotics have been the first-line treatment to control photobacteriosis outbreaks, although minimizing the use of antibiotics is a priority to avoid the development of resistant pathogen strains (Defoirdt et al., 2011) and to reduce the negative impact of drug residues on the consumer's health (Heuer et al., 2009) and on the environment (Kovalakova et al., 2020; Kumar et al., 2012).

Therefore, selective breeding programs for disease resistance play a key role in hindering the spread of pathogens, in order to achieve long-term control of the disease (Das and Sahoo, 2014) and because genetic improvement is cumulative and permanent (Doan et al., 2017). In addition, the improvement in disease resistance inherited by new generations may provide protection at the larval stage, when the immune system is typically not fully developed (Zapata et al., 2006). Selective breeding programs have been initiated in gilthead seabream to improve growth performance and morphology traits; other objectives (feed efficiency and product quality) have been established later (Perera et al., 2019), and different breeding programs for disease resistance are currently supported (Janssen et al., 2017; Massault et al., 2010; Palaiokostas et al., 2016).

Previous studies in seabream demonstrated that *Phdp* resistance showed an oligogenic-polygenic architecture and highlighted some associated QTLs (Aslam et al., 2014; Massault et al., 2010; Palaiokostas et al., 2016). Heritability of *Phdp* resistance has been found to be low to moderate, being higher when genomic information is used, and to be positively correlated with body weight (Antonello et al., 2009; Aslam et al., 2014; Massault et al., 2010; Palaiokostas et al., 2016).

Challenge tests are the most frequently used means to determine if a fish is resistant, the fish are subjected to a controlled infection, in a standardized environment, using one pathogen at a time. These criteria minimize possible variations caused by uncontrolled sources, maximize the reproducibility of the procedure, and improve the interpretation of data in terms of individual resistance to the pathogen (Ødegård et al., 2011). However, for biosecurity of infection reasons, infected fish cannot be used as selection candidates, but they can be used indirectly into a breeding program for the genetic evaluations of their relatives.

Useful biomarkers for identifying resistant animals could be immunological measurements, they are mainly components of the innate, or non-specific, immune system, such as antimicrobial activity (bactericidal and peroxidase activities) and of the adaptative, or specific, immune system, such as immunoglobulin M (IgM) levels. In fish, the innate immune response has been considered an essential component in combating disease incidents (Sunyer and Tort, 1995), acting as the first line of defense against foreign agents until the specific response is activated. Teleost fish possess a complete immune system and cellular and humoral responses with specificity and memory (Quade and Roth, 1997). To our knowledge, there has been no work about genetic variation for immunological markers in gilthead seabream, although studies have been conducted in other species, especially in salmonids and carp (Sahoo et al., 2011).

The aim of the present study was to estimate genetic parameters for *Phdp* resistance along with humoral immune markers, for the first time in gilthead seabream, at naïve conditions (peroxidase activity and bactericidal activity, IgM level), as well as their correlations between them and with body weight. Moreover, the estimated genetic parameters would also be considered for inclusion in the current commercial selective breeding program from which the fish of the experiment originated from.

1.2. Materials and Methods

1.2.1. Ethics statement

To ensure that animal welfare standards are maintained, anesthetic was used within the sampling procedure. All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU), the Bioethical Committees of the IEO (reference REGA ES300261040017) and the "Consejería de Agua, Agricultura y Medio Ambiente" of the Region of Murcia, Spain (approval number A13191103).

1.2.2. Animals

The experiment was carried out in gilthead seabream juveniles, which were obtained from two different broodstocks, belonging to the Spanish genetic breeding program PROGENSA®. The first broodstock (n = 133, 57♂ and 76♀) came from the Mediterranean Sea and was maintained in the Instituto Español de Oceanografía, Mazarrón, Murcia (IEO). It was never subjected to genetic selection (F0 generation), and hereinafter this broodstock is referred to as F0_MED. The second broodstock (n=51, 17♂ and 34♀) originally came from Andalusian coast Atlantic Ocean (ATL) and was maintained in the Instituto de Investigación y Formación Agraria y Pesquera, el Toruño, Puerto de Santa María, Andalusia (IFAPA). This broodstock was created after two genetic selection cycles as follows: The F1 offspring were evaluated in cages and ponds as described in Lee-Montero et al. (2013). For the ATL population, a total of 58 fish were selected after BLUP analysis as the F1_ATL broodstock by the estimated breeding value (EBV) for fork length at harvest (average +2.9%) and setting malformation EBV at zero. Thereafter, a second selection round was carried out and the F2 offspring were evaluated following the same experimental design as in the first selection round. In this selection round, the F2_ATL broodstock was selected by the harvest weight EBV (average 12.9% higher) setting malformation EBV at zero.

Each broodstock in its respective application centre was under a controlled photoperiod (8L:16D) to synchronize maturation; egg release was initiated at the beginning of December 2016. During that period, the animals were fed ad libitum by Vitalis Cal (Skretting), and egg production was monitored daily. When the total egg production was stabilized, eggs were collected by buoyancy on the same four consecutive days (4DL model) at each facility at the end of February 2017 to maximize family representation. Incubation was carried out in cylinder

conical tanks (1000 L) for 48 h at a density of 500–1000 larvae L-1. Water conditions were as follows: Temperature 19.0°C, salinity 34‰, and dissolved oxygen was 6.4 mg. L-1. A random larvae sample from F2_ATL was taken to IEO and located in one tank (5000L). Another larvae sample from F0_MED was placed in another tank (5000L). Both batches were reared in the conditions described by Chaves-Pozo et al. (2009). At 230 dph, 465 offspring of F0_MED and 530 offspring of F2_ATL were individually tagged in the abdominal cavity for individual identification with a Passive Integrated Transporter (PIT, Trovan Daimler-Benz), following the tagging protocol described by Navarro et al. (2006). Prior to the infection, fish with 250 dph of age from both populations, F0_MED and F2_ATL, were sedated with 20 µl/L of clove oil in sea water, and 100 µL of blood was extracted by puncture of the caudal vein to analyze immunological markers. The blood obtained was left to clot at 4°C for 16 hours and centrifuged at 10,000xg for 10 minutes at 4°C to obtain the serum that was frozen at -80°C until further analysis, as previously described (Cuesta et al., 2004; Hernández and Tort, 2003; Sunyer and Tort, 1995). The amount of blood extracted was found to be the maximum amount that could be extracted without risking the life of the specimens and minimizing handling losses. Even so, a 23% average mortality was recorded in the days after extraction, this was slightly higher among the F2_ATL fish (survivor fish n=368 F0_MED and n=325 F2_ATL). The fish were left to recover under open circuit culture conditions for at least 20 days, and then weighed and infected at 272 dph.

1.2.3. Bacteria culture

The *Photobacterium damselae* subsp. *piscicida* (*Phdp* strain PC-435.1, kindly provided by Dr. A.E. Toranzo) cultures were grown in soybean tryptone broth (TBS) at 20°C for 48 hours. The exponentially growing culture was washed three times with sterile phosphate buffered saline (PBS) by centrifugation at 2,700xg for 15 minutes at 4°C. The optical density of the bacterial suspensions was measured at 540 nm, and the number of colony forming units (cfu)/mL was calculated with a growth standard curve. The bacterial suspension was adjusted to $8x10^5$ cfu/mL.

1.2.4. Challenge test

When the fish were recovered, they were transported to the infection facilities, randomly distributed among five rectangular tanks of 200 L capacity. The tanks included an independent

recirculation system composed of a mechanical and biological filter, two aerators, a stabilization tank, and a submersible recirculation pump. The fish were intraperitoneally injected (i.p.) with 100 µL of PBS containing $8x10^5$ cfu/mL, a sublethal dose of *Phdp* ($8x10^4$ cfu/fish) for fish with a mean body weight of 15.01±5.47 g at 272 days post-hatching (dph). A control group (n=30 fish) injected with PBS alone was also placed in a similar tank with an independent recirculation system. Mortalities were recorded twice per day for the first three days and daily from day 4 onwards. After nine days of i.p., the fish overcame the infection as the cumulative mortality in the previous three days was fewer than three fish in all tanks. All the surviving fish were sacrificed using an excess of anesthesia (40 µL/L of clove oil in seawater).

1.2.5. Recorded traits

- Body weight

  At 272 dph, body weight (BW) was measured using scales accurate to 0.1 g.

- Resistance to disease: survival and days to death

Resistance to infection was measured as a binary trait of *Phdp* survival and days to death. The surviving fish were assigned the number 0 and the susceptible fish were given the number 1. Mortality rate was calculated as the number of dead fish divided by the total fish within the population. Mortality rate was also calculated for 12 intervals with a weight range of 2 g, from 6 to 30 g, for each population. Days to death were measured for nine days. The infection was considered finished after nine days, because the number of deaths accumulated in the previous three days was fewer than three in all tanks. When a fish died, a part of the tail fin was cut off and stored in ethanol for later DNA extraction.

- Humoral immune levels measurements

Natural peroxidase and total bactericidal activities were used as markers of innate humoral immune status and the level of total immunoglobulin (Ig) M as marker of adaptative humoral immune status in serum samples.

- ***Peroxidase activity***

The peroxidase activity levels in serum were measured according to a protocol previously described (Quade and Roth, 1997). Briefly, 5 µL of serum was diluted with 45 µL of Hank's buffer (HBSS) without Ca+2 or Mg+2 in flat-bottomed 96-well plates (Nunc) and mixed with 100 µL of 10 mM TMB solution containing 0.015 % H_2O_2 as substrate. The color-change reaction was stopped after 15 min of incubation by adding 50 µL of 2 M sulphuric acid, and the optical density (OD) was read at 450 nm using a plate reader (MultiskanGo, Thermo Fisher Scientific). Wells with HBSS but without a sample were used as blanks. Samples were run in triplicates. One unit was defined as the amount of activity producing an absorbance change of 1 and the activity was expressed as U.I./mL of serum.

- ***Total bactericidal activity***

The pathogenic marine bacteria Vibrio harveyi (Vh) (strain Lg 16/100) was grown in agar plates at 25°C in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1-2 mm were diluted in 5 mL of tryptic soy broth (TSB, Laboratorios Conda), cultured for 16 h at 25°C in an orbital incubator at 200-250 rpm and adjusted to 108 cfu/mL with TSB. The absorbance of bacteria cell cultures was measured at 600 nm and used to determine the concentration based on growth curves.

The antibacterial activity of serum was determined by evaluating its effects on the bacterial growth of Vh curves using a method previously described (Sunyer and Tort, 1995). Aliquots of 10 µL of the bacterial dilutions of Vh (1/10) were placed in flat-bottomed 96-well plates and incubated with 10 µL of serum for 2 h at room temperature. Then, 150 µL of TSB was added and the absorbance of the samples was measured at 620 nm every 30 min intervals for 36 h at 25°C. Samples with no bacteria were used as blanks (negative control). Samples without serum were used as positive controls (100 % growth or 0 % antibacterial activity). Total bactericidal activity was calculated as the percentage of bacterial growth inhibition per mL of plasma.

- ***Total levels of immunoglobulin M***

A direct ELISA was used to detect total IgM in gilthead seabream serum. For IgM detection, MaxiSorp 96-well plates (Nunc, Rochester, NY, U.S.A.) were coated with 100 µl of a 1:500 dilution of fish serum in carbonate / bicarbonate buffer pH 9.6 and incubated overnight at 4°C. After three washes of 5 min with 200 µl PBS containing 0.05% of Tween-20 (Sigma, PBS-T), the plates were blocked with 200 µl of PBS containing 3% of bovine serum albumin (BSA, Sigma) for 1 h at room temperature (RT). Subsequently, the washing steps were repeated and 50 µl of a monoclonal mouse anti- gilthead seabream IgM (Palenzuela et al., 1996) at the optimal dilution of 1:100 in PBS with 1% BSA was added, and plates were incubated for 1 h at RT. After washing the plate with PBT-T, 100 µl of anti-mouse-IgG-HRP (Sigma) at the optimal dilution of 1:1,000 in PBS with 1% BSA was added and incubated for 1 h at RT. After washing, the reaction was developed with 0.1 mM TMB with 0.025% H_2O_2. The reaction was stopped after 10 min of incubation at RT by adding 50 µL of 2 M sulphuric acid, and the OD was read at 450 nm with a microplate reader (MultiskanGo, Thermo Fisher Scientific).

1.2.6. Microsatellite Genotyping and parental assignment

The broodstock and offspring were genetically characterized. To this end, DNA was extracted from the caudal fin, conserved in absolute ethanol at room temperature using the *DNeasy kit* (QIAGEN®), and then kept at 4°C. Next, DNA quantity and quality were determined with a NanoDrop™ 2000 spectrophotometer v.3.7 (Thermo Fisher Scientific, Wilmington, U.S.A.). The multiplex SMsa1 (Super Multiplex *Sparus aurata*) was used as described in Lee-Montero et al. (2013) for genotyping the broodstock and offspring. The electropherogram was analyzed using Microsatellite analysis cloud (Thermo Fisher Scientific). Direct count of heterozygosity in the offspring of each population was calculated with the Excel package called Gene Alex (Peakall and Smouse, 2012). For the parental assignment the exclusion method as implemented in VITASSING (v.8_2.1) software (Vandeputte et al., 2006) was used. The number of fish assigned to a single couple was 272 for F2_ATL and 337 for F0_MED, with which the genetic parameters were estimated.

1.2.7. Statistical analysis

For phenotypical analysis

Numerical data for each trait were tested for normality and homogeneity of variances using SPSS® (v.25.0) (IBM Corp. Released, 2017) and were analyzed with two General Linear Models (GLMs):

1) $Y_{ij} = \mu + origin_i + e_{ij}$; for BW
2) $Yij = \mu + origin_i + b*BWj + eij$; for humoral immune markers and days to death (1)

in which Y_{ij} is an observation of an individual j from the origin i, μ is the overall mean, origin is the effect of the broodstock origin that produced both populations (i= F0_MED or F2_ATL), b is the regression coefficient between the analyzed variable and the covariate BW, and e_{ij} is a random residual error.

Since survival to infection is a binary trait, it was analyzed by logistic regression (SPSS® v25.0) including the origin effect as categorical covariate and the BW logarithm effect as numerical covariate, odds ratio (O.R.) and their confidence interval al 95% (CI_{95}) were calculated. The level of significant difference was set at $P < 0.05$.

For genetic analysis

Genetic parameters were estimated under a Bayesian approach using a bivariate mixed model. The model was,

$$Y = X\beta + Zu + e \quad (2)$$

Where:

Y is the recorded data on the studied traits,

β included the fixed origin effect for BW,

β included the covariate body weight for *Phdp* survival, days to death, and humoral immune biomarkers,

u is the random animal effect, and

e is the error.

This was performed using gibbs1f90 program for all traits except for disease resistance, which was considered a threshold trait, its genetic parameters (heritabilities and genetic correlations) were estimated in the underlying liability scale and analyzed with the program thrgibbs1f90 developed by Misztal et al. (2015). The estimates on the underlying liability scale assume that the susceptibility for the deformity is determined by an underlying liability that is distributed normally and inherited in a polygenic manner (Gjerde et al., 2005). The analysis was carried out between two traits each time. The following multivariate normal distributions were assumed a priori for random effects:

$$P(\beta) \sim k,$$

$$P(u|G) \sim (0, G \otimes A), \quad (3)$$

$$P(e|R) \sim (0, R \otimes I_e),$$

where A is the relationship matrix, and k is a constant,

$$G = \begin{bmatrix} \sigma_{U1} & \sigma_{U1,U2} \\ \sigma_{U2,U1} & \sigma_{U2} \end{bmatrix}, R = \begin{bmatrix} \sigma_{e1} & \sigma_{e1,e2} \\ \sigma_{e2,e1} & \sigma_{e2} \end{bmatrix}. \quad (4)$$

Bounded uniform priors were assumed for the systematic effects and the (co)variance components (G, A). A single chain of 200,000 iterations was run. The first 50,000 iterations of each chain were discarded, and samples of the parameters of interest were saved every five iterations. Density plots to represent posterior marginal distribution of heritabilities, posterior means (PM) and the 95% interval of the highest posterior density (HPD 95%) were obtained through R Development Core Team (2016). The magnitude of estimated heritability was established following the classification recommended by Cardellino and Rovira (1987), as low (0.05–0.15), medium (0.20–0.40), high (0.45–0.60) and very high (>0.65). The magnitude of correlation was established following the classification of Navarro et al. (2009), low (0–0.40), medium (0.45–0.55) and high (0.60–1), regardless of whether they were positive or negative.

1.3. Results

1.3.1. Phenotyping

Phenotypic data for BW and immunological markers in gilthead seabream juveniles from the two populations are shown in table 1. In addition, BW distribution was further analyzed in depth since the F2_ATL broodstock had been subjected to two selection rounds for growth (Figure 1). Juveniles from F2_ATL showed the heaviest BW, 27.7% heavier BW than juveniles from F0_MED. For BW distribution, F2_ATL showed a wider variability (70 % of fish were in the range from 10.5 to 23.7 g) than F0_MED (70 % of fish were in the range 9.9 to 17.1 g). The weight for the heaviest 30% of the fish was over 19.2 g for F2_ATL and 14.4 g for F0_MED. This threshold value was closer to the respective average for F0_MED since its data was more clustered.

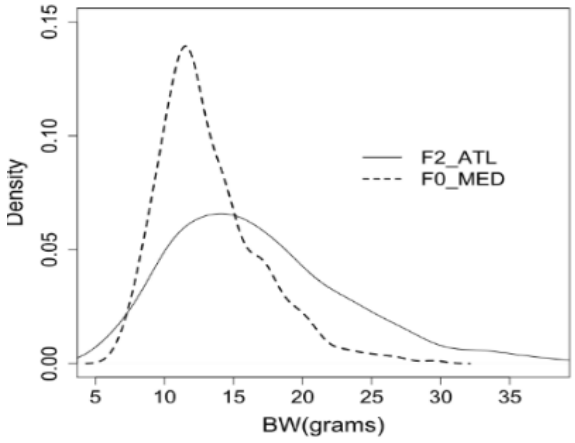


Figure 1. Body weight (BW) at 250 dph distribution for both populations. F2_ATL = F2 Atlantic Ocean population (n = 325) and F0_MED = F0 Mediterranean Sea population (n = 368).

Regarding the infection trial, mortalities were observed in both populations and started at the same time point (one day post-infection). A significant population effect was observed on mortality rate: F0_MED mortality risk was 1.48 (CI_{95} = [1.08 - 2.04]) higher than F2_ATL one. Mortality rates in the F2_ATL and F0_MED populations reached 29.10% and 38.42%, respectively (Figure 2). No mortalities were recorded in the control group. The mortality mainly happened in the first three days post-infection (93.6% in F2_ATL and 88.8% in F0_MED), on later days only one or two fish died, with the exception for day 5 in F0_MED, when 11 fish

died. The clinical signs observed in the infected fish were hemorrhaging around the bases of the fins and the urinogenital opening and along the pelvic and lateral fins and necrosis in the skin, producing severe skin lesions (Figure 7, in the introduction).

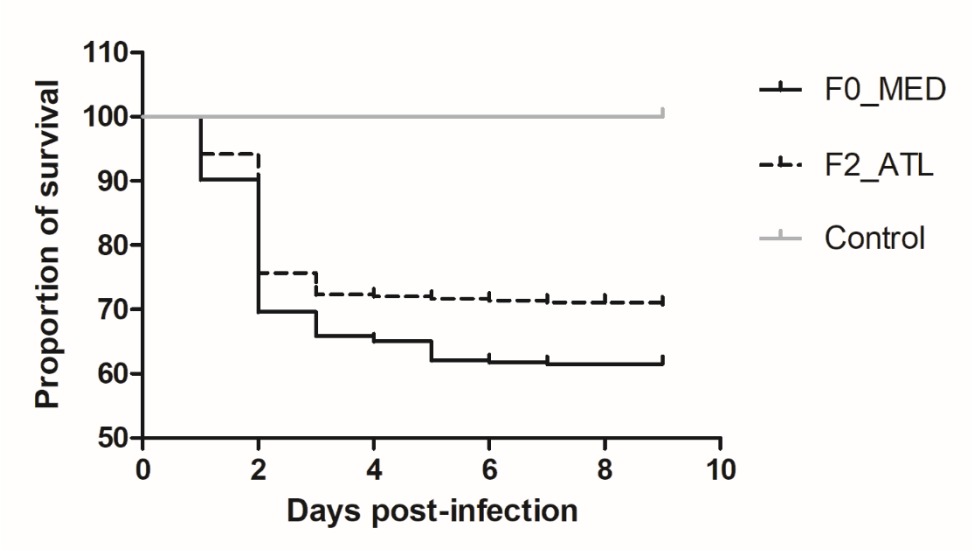
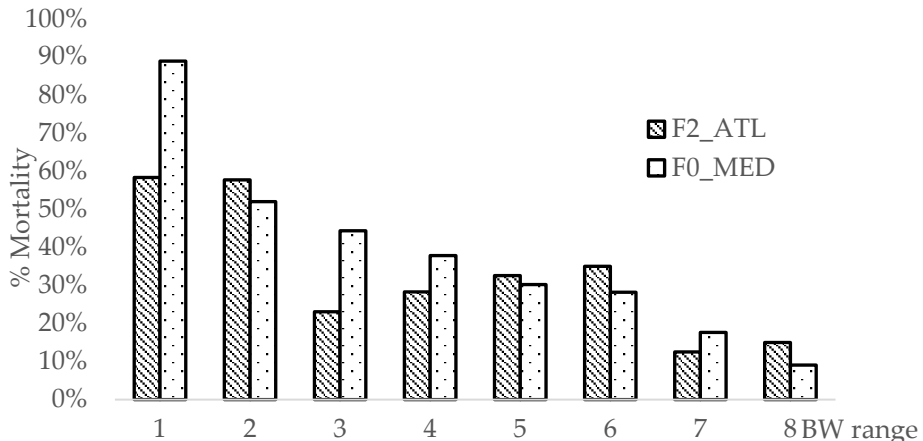


Figure 2. Kaplan-Meier survival curves showing the proportion of F0_MED and F2_ATL survivors after intraperitoneal injection with $8x10^4$ cfu/fish and a non-infected (control) group. F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population.

The BW effect on Mortality rate was significant, but this effect was not linear, the logarithmic transformation was needed to reveal that higher logBW meant lesser mortality risk (O.R = 0.68, CI95 = [0.48-0.96]). The average BW for dead fish was 13.4 g (standard error or s.e. 0.3) and for surviving fish was 15.8 g (s.e. 0.26), although this difference was small. The highest mortality rate occurred for weight from 6 to 10 g (Figure 3).



F2_ATL	7/12	15/26	9/39	13/46	14/43	14/40	4/32	3/20
F0_MED	8/12	26/50	47/106	31/82	13/43	11/39	3/17	1/11

Figure 3. Mortality rate upon infection depending on the broodstock origin and body weight (BW) represented by ranges in g: 1 (6.00-7.99g), 2 (8.00-9.99g), 3 (10.00-11.99g), 4 (12.00-13.99g), 5 (14.00-15.99g), 6 (16.00-17.99g), 7 (18.00-19.99g), 8 (20.00-21.99g). In the table below the graph, the first and second rows present the number of dead fish/total fish in each BW range and population. F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population.

In addition, disease survival was measured daily and days to death were recorded (Table 1). The average days to death was 2.2 (s.e. = 0.12), with no significant differences being observed between populations. The BW had no effect on days to death.

Regarding humoral immune markers, juveniles from F2_ATL showed much higher, more than double, peroxidase activity and slightly higher IgM levels than those from F0_MED. No significant differences were observed for bactericidal activity. The BW showed a negative effect for peroxidase activity, but only in F0_MED juveniles; it was not significant in F2_ATL.

Table 1. Phenotypic results (least square means ± standard error) for body weight and immunological markers for juvenile gilthead seabream from two populations.

Broodstocks origin[1]	**F2_ATL**			**F0_MED**			**Cov *BW***	
	n	**LSM**	**S.E.**	**n**	**LSM**	**S.E.**	**b**	**S.E.**
BW (g)	325	16.9[a]	0.28	368	13.2[b]	0.27	-	-
Days to death	93	2.13	0.12	141	2.23	0.10	0.019	0.019
Peroxidase activity (U.I./mL)	285	38.2[a]	1.65	359	15.7[b]	1.46	-0.308*	0.194
Bactericidal activity (%)	143	17.8	0.98	294	18.8	0.66	-0.014	0.099
IgM levels (O.D. 450 nm)	202	0.29[a]	0.006	316	0.26[b]	0.005	-0.001	0.001

[1] Broodstocks origin: F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population, BW = body weight, IgM = Immunoglobulin M, ab: different superscripts within each row indicate significant differences between origins ($P < 0.05$), b = regression coefficient for BA1, IgM levels, Peroxidase activity, Bactericidal activity, and days to death were adjusted to average BW 14.96, 14.77, 14.71, and 14.78 g, respectively, * = covariate was significant ($P < 0.05$), but it was only significant for F0_MED origin.

1.3.2. Microsatellite Genotyping and parental assignment

The breeders' contribution and the offspring assigned are shown in table 2. After assignment, an unequal breeder contribution was observed. In F2_ATL, nine out of 34 females produced 55% of the offspring although all the females contributed to the offspring, and two out of 17 males contributed 49% of the offspring and one male did not contribute any. Similarly, in F0_MED, eight out of 76 females contributed 46% of the offspring whilst 19 females did not produce any offspring, and 12 out of 57 males contributed 54% of the offspring; nine males did not contribute any. Pedigree construction using selected highly informative microsatellite markers yielded 54 full-sib families for F2_ATL with a mean of 3.52 sibs (range 2-17 sibs). Conversely, in the case of F0_MED, it produced 48 full-sibs with a mean of 2.85 sibs (range 2-12 sibs). 57♂ and 76♀).

Table 2. Parental assignment.

Broodstocks origin[1]	**F0 MED**		**F2 ATL**	
	Males	**Females**	**Males**	**Females**
Total number of breeders	57	76	17	34
Breeders that contributed	48	57	16	34
Offspring assigned (%)	97.54		87	
Offspring assigned to only one pair of parents (%)	92		83.9	

[1] Broodstocks origin: F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population

Regarding the study of genetic variation considering the microsatellites genotypes, high heterozygosity was observed in both populations; this was 0.75 and 0.78 for F0_MED and F2_ATL, respectively.

1.3.3. Genetic parameters

- Heritability

Heritability for BW was moderate (PM=0.20 and HPD = [0.08-0.30]). For *Phdp* survival, heritability was moderate (0.32 [0.15-0.45]); however, it was low for days to death (0.05 [0.02-0.14]). Regarding humoral immune markers, it was moderate 0.30 [0.16–0.48] for peroxidase activity, and low (0.10 [0.01–0.23] and 0.09 [0.00-0.19], respectively) for IgM levels and bactericidal activity (Figure 4).

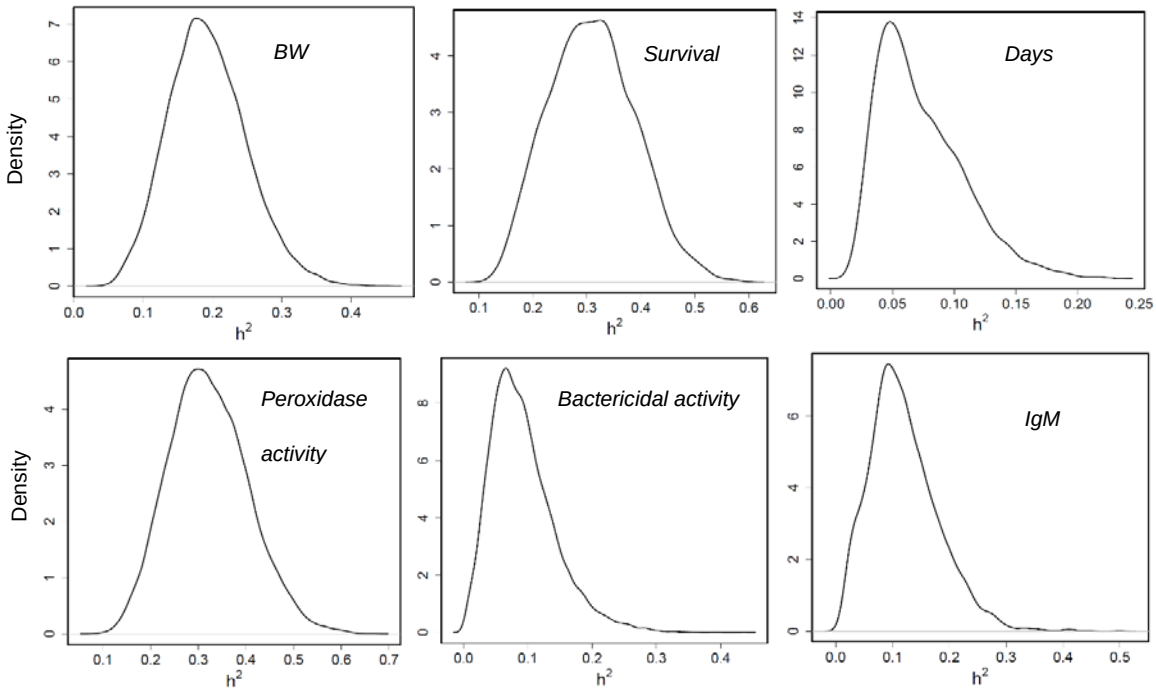


Figure 4. Posterior marginal distribution of heritabilities of body weight (BW), *Photobacterium damselae* resistance, days to death and immunological markers (peroxidase activity, bactericidal activity, and immunoglobulin M (IgM) levels) gilthead seabream. h2=heritability. All measure with 609 data, except to bactericidal activity with 457 data and IgM with 566 data.

- Genetic correlations

Weight showed a very high (close to 1) and positive favourable genetic correlation with *Phdp* survival and days to death. Accordingly, genetic correlations of *Phdp* survival and days to death were highly and positively correlated. Genetic correlation between weight and peroxidase activity and IgM levels tended to be positive, although the estimate lacked accuracy. On the contrary, the genetic correlation between weight and bactericidal activity was high but negative (close to -1) (Figure 5). For humoral immune markers there were fewer data, especially for IgM levels and bactericidal activity, therefore the genetic correlations should be considered with caution. Furthermore, the posterior distributions of the genetic correlations estimated from the Bayesian analysis appear to have significant skewness and trailing ends, which may be because the programs delete incorrect values greater than one.

Genetic correlations between *Phdp* survival and humoral immune markers were very high, positive with peroxidase activity and negative with IgM levels and bactericidal activity. The same pattern was observed for days to death with peroxidase activity and bactericidal

activity, although the correlation between days to death and IgM levels was less accurate and positive. Peroxidase activity and IgM levels were negatively correlated.

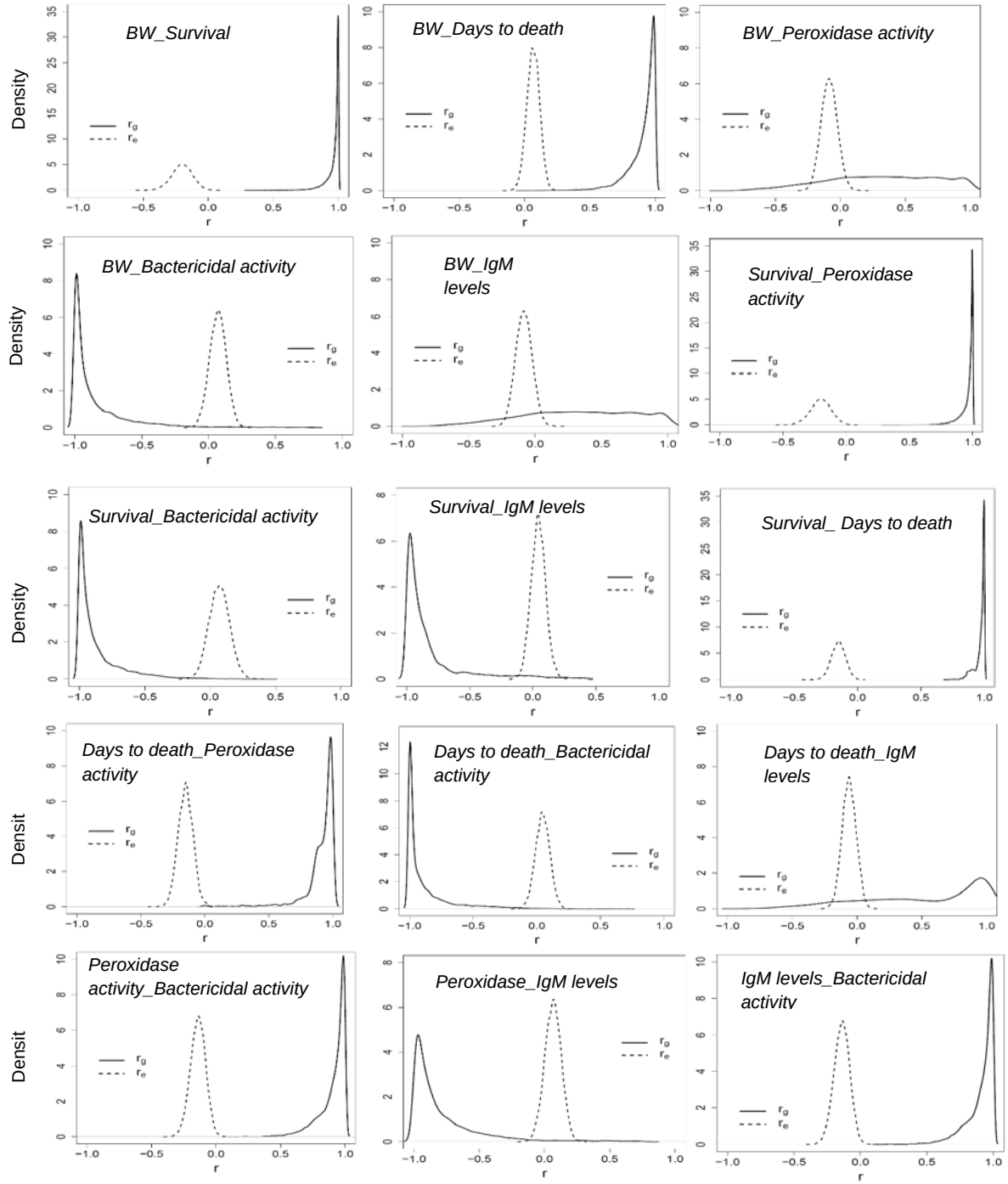


Figure 5. Posterior marginal distribution of genetic correlations of body weight (BW), Photobacterium damselae resistance, days to death and immunological markers (peroxidase activity, bactericidal activity, and immunoglobulin M (IgM) levels) gilthead seabream. rg = genetic correlation; re = residual correlation. All measure with 609 data, except to bactericidal activity with 457 data and IgM with 566 data.

1.4. Discussion

The F2_ATL population was the offspring of the second generation of breeders from the PROGENSA project (PROGENSA®, http://www.progensa.eu) selected to increase the growth rate and to decrease deformities, while the F0_MED population came from a new broodstock from Southern Mediterranean Sea that has never been subjected to selection. At the beginning of the PROGENSA project (2009), zero generation, this Atlantic (F0_ATL) population was studied together with other Northern Mediterranean Sea Populations. At that time (García-Celdrán et al.,2015), higher BW was observed for the Mediterranean populations than for the Atlantic Ocean population, contrary to the current work. However, we cannot compare the selection response for growth with these previous results of ATL population since weight was not measured at exactly the same ages and rearing conditions also differed. When these populations were genotyped for SMSa1 multiplex, no effects of population or selection process were observed when F2_ATL was compared with F0_ATL heterozygosity (Ho = 0.72), revealing that for the moment genetic variability for these microsatellites is being kept. Heritability for BW (0.2) was within the range estimated by other authors (Carballo et al., 2020; García-Celdrán et al., 2015). Thus, Carballo et al. (2020) showed BW heritabilities of 0.18 and 0.06 in two batches with fish at 80 dph and at 140 dph, respectively. García-Celdrán et al. (2015) reported heritabilities of 0.11 and 0.25 at 163 and 690 dph, respectively, and they pointed out that heritability estimates for growth traits increased with age when they compared juveniles with commercial size fish.

Regarding disease survival, a population effect was revealed on mortality rate in contrast with Antonello et al. (2009) and Palaiokostas et al. (2016) who studied *Phdp* survival for different Adriatic and Atlantic broodstocks of gilthead seabream. In our work, the F2_ATL population had been subjected to a selection process to improve body weight, and heavier fish were more resistant.

In our work, days to death was around 2.2 days when fish with a mean body weight of 15.01±5.47 g at 250 dph were challenged by intraperitoneal injection of a dose of the $8x10^4$ cfu/fish and around 66% of the fish survived. In Antonello et al. (2009), when fish of a mean body weight of 0.4-0.6 g were infected by immersion in sea water containing $1x10^5$ cfu of *Phdp*/100L, the mortality peaks were at day 7 and at day 11 post challenge and most fish died. Palaiokostas et al. (2016), in the same conditions as Antonello et al. (2009) found three distinct peaks at days 7, 11, and 15 during challenge, the same as Massault et al. (2010) with a dose $3x10^6$ cfu of *Phdp*/100L. Aslam et al. (2014) with fish 3-4 grams in weight and 120 dph infected

by intramuscular inoculation with 1000 cfu *Phdp*, observed 37% mortality and only one peak at day 5. Therefore, injection was more effective for infection and only one peak was observed.

Regarding genetic variation, heritability was moderate for survival on the liability scale and low for days to death. Our results are in the range of those of Antonello et al. (2009), who observed lower heritability for days of survival post *Phdp* challenge (0.12 ± 0.04), defined as a continuous trait, while it ranged from 0.45 ± 0.04 to 0.18 ± 0.08 for the binary trait dead/alive on a specific day. Palaiokostas et al. (2016) showed that heritability of surviving days was 0.22 (HPD: 0.11–0.36) and 0.28 (HPD: 0.17–0.40) using the pedigree and the genomic relationship matrix, respectively. Aslam et al. (2014) showed similar heritabilities for disease resistance (dead/survive phenotype) and days to death (~ 0.32) with the pedigree or the genomic information. Nevertheless, it seems that heritability estimates of mortality traits are frequency dependent, with maximal values reported at intermediate mortality levels (Bishop and Woolliams, 2010).

Therefore, we can conclude that the results of this study, along with the results of other studies, demonstrate the existence of a genetic component for disease resistance. A relatively good response to selection should be expected when breeders are selected through the offspring to improve *Phdp* resistance. However, the main flaws in this process are that a longer time elapses to assess a breeder and the infected offspring must be slaughtered for biosecurity of infection reasons.

As far as the genetic correlations are concerned, weight was positive and highly correlated with *Phdp* survival and days to death, in accordance with Antonello et al. (2009) when the correlation between *Phdp* survival and body length was estimated. However, different results have appeared for other species and other bacterial diseases. Thus, when columnaris disease (CD) caused by *Flavobacterium columnare* in Rainbow trout (*Oncorhynchus mykiss*) was studied, the data showed negative correlations between these two traits (Evenhuis et al., 2015).

Regarding the humoral immune markers, we analyzed the peroxidase activity and total bactericidal activities and total IgM levels at naïve immune status and found that the population effect was only observed for peroxidase activity, being higher for F2_ATL. Peroxidase activity and IgM levels in skin mucus and serum increased when fish were exposed to stressful conditions (Guardiola et al., 2016). There is a link between stress-immunodepression-disease susceptibility, thus developing lines of highly resilient farmed fish (ability to maintain productivity when coping with different environmental challenges) might be a strategy to improve disease resistance (Janssen et al., 2017) or reciprocally, improving disease resistance

in fish could lead them to being able to cope with environmental challenges. In our study, F2_ATL showed the highest peroxidase activity, and the lowest overall mortality rate. Therefore, the higher peroxidase activity is likely to make the fish better able to cope with the disease. Lund et al. (1995) studied genetic variations for immunological markers (Lysozyme activity, hemolytic activity, total IgM level, and levels of antibodies measured after immunization) in Atlantic salmon challenged to *Aeromonas salmonicida* and *Vibrio salmonicida;* those authors found significant genetic variation in lysozyme activity, as well as an apparent genetic association between low lysozyme activity and high survival rates. Low heritabilities and low correlations with survival were estimated for all the other immune markers (Lund et al. 1995). Sahoo et al. (2011) in two lines of Indian major carp *Labeo rohita* showed higher activity of the respiratory burst of blood phagocytes and serum myeloperoxidase and higher ceruloplasmin level were significant in the resistant line compared to the susceptible line when these biomarkers were measured after infection.

In our work, humoral immune markers were measured before infection, and moreover we found only moderate heritability for peroxidase activity that showed a high genetic correlation with disease resistance. However, to our knowledge, this is the first study to demonstrate that some humoral immune markers at naïve conditions could be used as a selection criterion for the breeders to improve their disease resistance, and that such a selection could be accomplished using their own phenotypical data instead of their offspring. Some humoral immune markers, such as peroxidase and lysozyme activities and IgM levels or a combined index of them, along with the weight of the fish and the absence of deformities, could be included in a breeding program to breed fish that are capable of coping with diseases and environmental challenges but also express good performance for production traits. Such fish would enjoy better animal welfare and be characterized as proactive fish or "active coping" (Castanheira et al., 2015; Ferrari et al., 2015).

Other studies have investigated the genes controlling disease resistance. Dios et al. (2007) investigated different gene expression in the brain of gilthead seabream infected with nodavirus, and Fjalstad et al. (2003) pointed out some advances in transgenic salmon including the rainbow trout lysozyme gene. Future research should be continued into the establishment of a breeding program to improve disease resistance and performance traits and searching for the genes or Quantitative Trait Loci (QTL) which control disease resistance.

1.5. Conclusions

Our results reveal, for the first time, that some humoral immune markers (peroxidase activity) at naïve conditions show moderate heritability. In addition, body weight is genetically and positively correlated with *Phdp* survival. Therefore, an alternative breeding program in gilthead seabream (*Sparus aurata* L.) is suggested to select fish for their own performance that consider increasing weight, reducing deformities and including humoral immune markers at naïve status, such as increasing peroxidase activity, to breed fish that are capable of dealing with diseases and environmental challenges and show good performance on other production traits.

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2.0. Abstract paper 2:

Humans require essential fatty acids in their diet and marine fish are a source of them, especially omega3 fatty acids that present high benefit on diverse vascular diseases and the immune system. Breeding programs in gilthead seabream usually include growth as first criterion in the selection process of the fish. However, it could lead to fish with higher fillet fat content and a fatty acid profile with less polyunsaturated fatty acids percentage. The study of the genetic parameters of the fatty acid profile in sea bream fillet was carried out in a population that was reared to commercial weight in a cage in the Mediterranean Sea. Samples were taken from those individuals at the beginning and at the end of fattening to determine body weight and total length. After the fish had been slaughtered, different meat quality variables (protein, collagen, fat, and moisture) and the fatty acid profile of the fillet were analyzed. Gilthead sea bream fillet showed a high percentage of monounsaturated fatty acids (MUFA, 44.4%), with 18:1n9c (33.6%) standing out, followed by the percentage of polyunsaturated fatty acids (PUFA, 29.8%), with 22:6n3 (6.8%) standing out, and finally the percentage of saturated fatty acids was 28.3%, with 16:0 being the majority. Medium heritability was observed for growth traits and meat quality variables, except for collagen, which showed low heritability. Regarding the acid profile, medium heritability was observed for 18:1 fatty acid, for MUFA and for ration3/n6; and low for 22:6n3. In addition, a negative phenotypic correlation was observed between fillet content and percentage of PUFA. Therefore, a selection for increased gilthead sea bream weight accompanied by an increase in fillet fat content may lead to a decrease in PUFA. From the consumer's point of view, it may be interesting to include the fatty acids profile of the fillet as a selection criterion because of their role in cardiovascular diseases and from the animal's point of view because of their relationship with its immune system.

Paper 2. Genetic Analysis of the Fatty Acid Profile in Gilthead Seabream (*Sparus aurata*)

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Simple Summary: Humans require essential fatty acids in their diet and marine fish are a source of them, especially omega3 fatty acids that present high benefits on diverse vascular diseases and the immune system. Breeding programs in gilthead seabream usually include growth as the first criterion in the selection process of the fish. However, that could lead to fish with a higher fillet fat content and a fatty acid profile with a lower polyunsaturated fatty acids percentage. Fillet fat content and its fatty acids profile have been revealed as heritable traits. Therefore, further studies to go deeper in the selection process are advisable.

Abstract: The gilthead seabream is one of the most valuable species in the Mediterranean basin both for fisheries and aquaculture. Marine fish, such as gilthead seabream, are a source of n3 polyunsaturated fatty acids, highly appreciated for human food thanks to their benefits on the cardiovascular and immune systems. The aim of the present study was to estimate heritability for fatty acid (FA) profile in fillet gilthead seabream to be considered as a strategy of a selective breeding program. 399 fish, from a broodstock Mediterranean Sea, were analysed for growth, flesh composition and FA profile. Heritabilities for growth traits, and flesh composition (fat, protein, and moisture content) were medium. Heritability was moderate for 14:0, 16:0 and 18:1n9 and for sum of monounsaturated FA and n6/n3 ratio, and it was low for 20:1n11 and 22:6n3 and the ratio unsaturated /saturated FA. Breeding programs in gilthead seabream usually include growth as the first criterion in the selection process of the fish. However, other quality traits, such as fillet fat content and its fatty acids profile should be considered, since they are very important traits for the consumer, from a nutritional point of view and the benefits for the health.

Keywords: Fatty acid profile; heritability; Gilthead Seabream; Body Weight; Moisture; Fat; Collagen; Protein.

2.1. Introduction

The gilthead seabream is one of the most valuable species in the Mediterranean basin both for fisheries and aquaculture. Total production in Mediterranean countries reached 253,000 metric tons in 2019. The three most important countries producing gilthead sea bream were Greece, Turkey and Spain, in that order (APROMAR, 2020) . In the Mediterranean area, notable success has been achieved in the production of diverse species, such as sea bream and

bass. Now that production technologies have been established, interest has been redirected to increasing the quality of the product offered (European Aquaculture Production Report, 2014).

Fatty acids (FA), especially n3 polyunsaturated fatty acids (n3 PUFA), eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic acids (DHA, 22:6n3), are recognized as being beneficial for human health, controlling a wide range of human pathologies including cardiovascular, diabetes, rheumatoid arthritis, osteoporosis, asthma, cognitive decline, neurological dysfunction and possible cancers, whilst also playing an important role in neural development and in the immune and inflammatory processes (Augustsson et al., 2003; Mccullough et al., 2002; Simopoulos, 2008; Tocher, 2015). Arachidonic acid (20:4n6, ARA), EPA and DHA are considered as the precursors for the synthesis of eicosanoids, regulators of cell signaling and gene expression and the most powerful modulators of cell membrane fluidity (Calder, 2006; Yaqoob and Calder, 2007). Alteration in the PUFA content of the immune cells was demonstrated to be associated with the alteration of non-specific immunity (e.g. phagocytosis, respiratory burst and serum lysozyme), specific immunity (e.g. antibody production and resistance to pathogens), eicosanoid production and immune related gene expression (Chen et al. 2016)

Humans, and probably all vertebrates, require essential FA in their diet that cannot be biosynthesised or interconverted, such us 18:2n6 (linoleic acid, LA) and α18:3n3 (αlinolenic acid, ALA) PUFA (Burr and Burr, 1930; Rivers and Frankel, 1981; Sciences, 1993). These essential fatty acids are primarily derived from plants that should be included in the vertebrates' diet. LA and ALA have vital functions in themselves, and in turn act as precursors for the long chain PUFA (LCPUFA) ARA, EPA and DHA. Their biosynthesis from LA and ALA can be carried out by mammals, although the process of EPA and particularly DHA biosynthesis from ALA is very low in humans and in marine fish (Graham and Calder, 2005; Tocher, 2015).The biosynthetic pathway involves consecutive desaturation and elongation reactions that convert LA to ARA and ALA to EPA and DHA. The two main enzyme families involved in these conversions are the elongases of very long fatty acids (Elovl) and the fatty acyl desaturases (Fad) (Tocher, 2015). The EPA pathway in teleost fish is often incomplete, primarily due to a lack of Δ5 desaturase, and so synthesis of EPA from ALA is not possible in many, particularly marine, carnivorous species (Tocher, 2010). However, the DHA pathway from EPA is probably functional in most teleost fish, including marine species, at least in some tissues.

Modern Western diets have an excess of n6 PUFA, primarily LA, and because n6 FA and n3 FA cannot be interconverted in vertebrates, this has led to an increase in the tissue ratio of n6 to n3 LCPUFA, linked to cardiovascular, inflammatory, and neurological problems

(Calder, 2006). One way of addressing this n6/n3 imbalance is to increase the levels of n3 PUFA and especially n3 LCPUFA in the diet of humans. Marine fish, such as the gilthead seabream (*Sparus aurata*), are a source of LCPUFA (Senso et al., 2007). However, vegetable feed in the fish diet has increased in recent years. Fillets of gilthead sea bream fed diets rich in plant oils show increased levels of LA and ALA, with a concurrent decrease in EPA and DHA (Benedito-Palos et al., 2008; Izquierdo et al., 2005).

Dietary and fillet FA composition are closely associated. However, Ballester-Lozano et al. (2011) observed that the FA composition (%) depends not only on the diet but also on the fillet lipid content (FLC). In general, monounsaturated FA (MUFA) increased with the increase of FLC, whereas the trend for saturated FA (SFA) and PUFA was the opposite. In the case of ARA and DHA, they decreased when the FLC increased. Thus, FLC could partly explain saturated, monounsaturated and some polyunsaturated (ARA and DHA) FA but not LA, ALA, EPA and docosapentaenoic acid (22:5n3, DPA). This is likely due to marine fish showing a limited ability to convert C18 FA into LCPUFA of n6 and n3 series (Sargent et al., 2002; Tocher, 2003). In addition, while triacylglycerols (TAG) are in fat deposits, and usually contain MUFA (C16-C18); polar lipids, in membrane cells are composed mainly of phospholipids that accumulate LCPUFA (Sushchik et al., 2020). An FLC increase is related to a higher amount of fat deposits and, concurrently, a higher proportion of TAG and MUFA and a lower proportion of polar lipids and LCPUFA.

Other factors besides diet (e.g., genotype, gender, age, and production system) have a significant influence on the fillet lipid level and thus on the FA composition of most animals (Makhutova and Stoyanov, 2021).

Hence, advances in breeding programs are essential to contribute to the profits and competitiveness of the companies, as well as to improve the quality of product, including its fatty acid profile.

Selective breeding programs have been initiated in gilthead seabream to improve growth performance and morphology traits; other objectives (feed efficiency, product quality and disease resistance) have been considered later (Ballester-Lozano et al., 2011; Ofori-Mensah et al., 2020; Perera et al., 2019; Vallecillos et al. 2021). Heritabilities, genetic and phenotypic correlations between different traits are key indicators in the success of such breeding programs.

To date, there have been few heritability studies in terms of the fillet fat content in gilthead seabream, in which medium heritability in fish and medium correlation with respect to weight were observed (Elalfy et al., 2021; García-Celdrán et al., 2015b). In addition, to the best of our knowledge, there has been no work about FA genetic variation in sea bream, but it has

been studied in Atlantic salmon (Leaver et al., 2011) and in Nile Tilapia (Nguyen et al., 2010). Flesh n3 LCPUFA composition was a highly heritable trait in Atlantic salmon (Leaver et al., 2011) however individual FA heritability varied from zero to medium in Nile tilapia (Nguyen et al., 2010). In Atlantic salmon, families with a high percentage of n3 LCPUFA in flesh showed higher expression of lipid transport genes, cell cycle and growth-related genes and increased activity of a transcription factor, hepatic nuclear factor 4α (HNF4 α). Dong et al. (2018, 2016) demonstrated that HNF4α is a transcription factor of the vertebrate *Fad* gene involved in the transcription regulation of LCPUFA biosynthesis. Therefore, it is nonetheless a sensible strategy to select for this trait to improve it and optimise the efficiency of n3 LCPUFA metabolism and flesh levels, irrespective of likely dietary levels.

The aim of the present study was to estimate genetic parameters for the FA profile in fillet sea bream, for the first time in gilthead seabream, to be considered as a strategy in a selective breeding program.

2.2. Materials and Methods

To ensure that animal welfare standards are maintained, anaesthetic was used within the sampling procedure. All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU) and, whenever necessary, fish were anesthetized.

2.2.1. Fish and rearing conditions

For growth, flesh composition (fillet fat, moisture, protein, and collagen percentages) and FA profile were analysed for 399 gilthead seabream fish. The fish came from a broodstock (n = 133; 57 males and 76 females) that had been captured in the Mediterranean Sea and maintained in Instituto Español de Oceanografía, Mazarrón, Murcia (IEO). The broodstock had never been subjected to genetic selection.

In the broodstock, the female/male ratio was approximately 2:1 in the tanks, they were under a controlled photoperiod (8L:16D) to synchronize maturation; and egg release was initiated at the beginning of February 2016. During that period, the animals were fed on Vitalis Cal (Skretting), and egg production was monitored daily. When the total egg production stabilized, one egg batch was established at the end of April 2016. Therefore, eggs from the broodstock were collected and pooled for four consecutive days (4DL model) to maximize

family representation. Incubation was carried out in cylindrical conical tanks (1000 L) at a density of 500–1000 larvae/L. Water conditions were as follows: Temperature 19.0°C, salinity 34‰, and dissolved oxygen was 6.4 mg/L.

At 251 days post-hatching (dph), the fish were individually tagged in the abdominal cavity for individual identification with Passive Integrated Transporter (PIT; Trovan Daimler-Benz), following the tagging protocol described by Navarro et al. (2006); initial body weight (BW_{251dph}) and total length (TL_{251dph}) were measured; and a sample of caudal fin was collected and preserved in absolute ethanol at room temperature for future DNA extraction. Ten days later, the fish were moved to the facilities of the company Servicios Atuneros del Mediterraneo S.L. (San Pedro del Pinatar, Murcia, Spain), where they were reared in a cage in the Mediterranean Sea under intensive conditions: a cage 11 meters in diameter which is anchored at a depth of 38 meters in the Mediterranean Sea (average water temperature = 18.2 ± 0.9 ºC, dissolved oxygen: 7.4 mg/l, 100% oxygen-saturation, salinity: 37.9‰; data estimated from open sea conditions). Fish were fed over the course of the study with extruded pellets (Dibaq S.A, Fuentepelayo-Segovia, Spain), with two different commercial diets. The first 15 months diet D4 was used (46.5% protein, 19% fat, 7% ashes, 2.75% cellulose, 17.9 MJ/kg digestible energy), and subsequently when the fish were around 220 g in weight, they were fed diet D6 (44% protein, 20% fat, 7.17% ashes, 3.07% cellulose, 17.6 MJ/kg digestible energy) until slaughter time. The FA composition of each diet was analysed in duplicate; the mean is shown in Table 1.

Table 1. Fatty acid (FA) composition of the diets (% total FAME), mean ± standard error.

FA	D4	D6
14:0	2.91 ± 0.09	1.89 ± 0.04
16:0	15.5 ± 0.11	11.4 ± 0.01
18:0	5.16 ± 0.34	4.31 ± 0.02
SFA	24.3 ± 0.09	17.5 ±0.08
16:1	4.05 ± 0.08	2.66 ± 0.00
18:1*	29.9 ± 0.18	42.9 ± 0.30
C 20:1	3.03 ± 0.08	2.38 ± 0.08
C 22:1	2.05 ± 0.32	1.17 ± 0.15
MUFA	39.2 ± 0.13	49.7 ± 0.54
18:2n6	18.9 ± 0.21	20.1 ± 0.31
18:3n3	4.61 ± 0.26	6.05 ± 0.03
20:4n6	1.18 ± 0.59	0.00 ± 0.00
20:5n3	4.58 ± 0.03	2.81 ± 1.40
22:6n3	6.19 ± 0.52	4.01 ± 0.06
PUFA	36.4 ± 0.04	32.6 ± 0.45

SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids . 18:1* refers only to n9c, because n9t was almost null.

At harvest size (980 dph), fish were slaughtered by immersion in ice cold water (hypothermia); final body weight (BW_{980dph}) and total length (TL_{980dph}) were measured. Fish were manually skinned and filleted without including the nape and the belly flap. Two pieces of fillet were vacuum packaged and frozen at -80ºC for further analysis.

2.2.2. Flesh composition quality

One piece of fillet from each fish was homogenized and analysed by indirect method of near-infrared spectroscopy (near infrared spectroscopy, NIR), using FOODSCAN LAB equipment (FOSS, Denmark), to obtain total collagen of the muscle, and chemical components of the muscle (fat, moisture, and protein), as a percentage of flesh.

2.2.3. Chromatographic analysis of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were prepared using a solution of KOH in methanol (IOfS, 2002), 17:0 acid was used as internal standard FA, then separated and analysed by gas chromatography. Analyses were performed on a 6890-gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a GERSTEL MultiPurpose Sampler (MPS2) and a mass spectrometer 5975 with a hyperbolic quadrupole (Agilent Technol.). Extract (0.8 µl) of FAME was injected and separated on a DB-23 capillary column (Agilent Technologies) of 60 m (length) x 0.25 mm (internal diameter) x 0.25 µm (film) in constant pressure mode.

Chromatographic-grade helium was used as the carrier gas. The temperature of the injector was 240℃. The inlet operated in split mode with a split ratio of 1:20. The initial oven temperature was 50℃ which was held for 1 min, then increased to 175℃ at 25℃ per min and thereafter increased to 235℃ at 4℃ per min, with a holding time of 10 min. Mass spectra were collected in the scan range m/z 40-400. The measurements were performed using an electron bombardment ion source with electron energy of 70 eV. The transfer line, source, and quadrupole temperatures were set at 280, 230, and 150℃, respectively. The chromatograms and mass spectra were evaluated using the ChemStation software (G1791CA, Version D.03.00, Agilent Technol.). Peaks were identified by comparison of retention times with FAME standards (Supelco 37 Component FAME mix, Sigma Aldrich, USA) and their mass spectra. The individual FAs are expressed as a percentage of the total FA detected.

2.2.4. Microsatellite Genotyping and parental assignment

The broodstock and offspring were genetically characterized. To this end, DNA was extracted from the caudal fin using the DNeasy kit (QIAGEN®), and then kept at 4°C. Next, DNA quantity and quality were determined with a NanoDrop™ 2000 spectrophotometer v.3.7 (Thermo Fisher Scientific, Wilmington, U.S.A.). The multiplex SMsa1 (Super Multiplex *Sparus aurata*) was used as described in (Lee-Montero et al., 2013) for genotyping the broodstock and offspring. The electropherogram was analysed using Microsatellite analysis cloud (Thermo Fisher Scientific). Direct count of heterozygosity in the offspring was calculated with the Excel package called Gene Alex (Peakall and Smouse, 2012). For parental assignment, the exclusion method as implemented in VITASSING (v.8_2.1) software (Vandeputte et al., 2006) was used. The number of fish assigned to a single couple was 399 and they were used to estimate genetic parameters.

2.2.5. Statistical analysis

All data were tested for normality and homogeneity of variances using SPSS (v.25.0, IBM Corp, 2017). For growth trait (BW and TL) arithmetic means and standard errors were calculated.

Flesh composition (fillet fat, moisture, protein, and collagen percentages) and FA profile were analysed with the following General Linear Model (GLM):

$$Y_{ij} = \mu + b*covariate_j + e_{ij} \quad (1)$$

in which,

Y_{ij} is an observation of an individual j from the origin i,

μ is the overall mean,

b is the regression coefficient between the analysed variable and the covariate *BW* for flesh composition or fillet fat percentage for FA profile,

e_{ij} is a random residual error.

The level of significant difference was set at $P < 0.05$.

Genetic parameters were estimated under a Bayesian approach using a bivariate mixed model. The model was,

$$Y = X\beta + Zu + e \quad (2)$$

Where *Y* is the recorded data on the studied traits, *β* includes covariate body weight (not included for BW and TL traits), *u* the random animal effect and *e* the error. This was performed using gibbs3f90 program for all traits, as developed by Misztal et al. (2015). The analysis was carried out between two traits each time. The following multivariate normal distributions were assumed a priori for random effects:

$$P(\beta) \sim k;$$

$$P(u|G) \sim (0; G \otimes A); \quad (3)$$

$$P(e|R) \sim (0; R \otimes I_e);$$

where A is the relationship matrix and k is a constant,

$$G = \begin{bmatrix} \sigma_{U1} & \sigma_{U1,U2} \\ \sigma_{U2,U1} & \sigma_{U2} \end{bmatrix}, R = \begin{bmatrix} \sigma_{e1} & \sigma_{e1,e2} \\ \sigma_{e2,e1} & \sigma_{e2} \end{bmatrix}. \quad (4)$$

Bounded uniform priors were assumed for the systematic effects and the (co)variance components (G, A). A single chain of 200,000 iterations was run. The first 50,000 iterations of each chain were discarded, and samples of the parameters of interest were saved every five iterations. Density plots to represent posterior marginal distribution of heritabilities, posterior

means (PM) and the 95% interval of the highest posterior density (HPD 95%) were obtained through R Development Core Team (2020).

2.3. Results and discussion

2.3.1. Phenotyping

The phenotypic results for growth at 251 dph and 980 dph (BW and TL), flesh composition (fat, collagen, moisture, and protein percentages) and FA profile at 980 dph in gilthead seabream are shown in Tables 2 and 3.

Regarding flesh composition, the fat percentage was high in comparison with that found by other authors (Elalfy et al., 2021; García-Celdrán et al., 2015b) who observed less fillet fat percentage when BW was lower in García-Celdrán et al. (2015b) 4.64% for BW_{690dph} = 271g, in Elalfy et al. (2021) 6.55% for BW_{700dph} = 313 g). However, when fish were raised in an estuary (Elalfy et al., 2021) and reached higher BW_{700dph} (440g), the fillet fat percentage increased (8.71%). In addition, a pronounced seasonality has been observed on fillet fat that reached a maximum with the replenishment of body fat stores in early autumn (Ballester-Lozano et al., 2011) when our fish were slaughtered. In addition, BW had a positive significant effect on fat percentage, and this effect was less pronounced for collagen percentage (when fish weight increased 100g the fat percentage increased 0.6% and collagen percentage decreased 0.1%). Contrary to the fat, in our study the moisture percentage was low in comparison with that in García-Celdrán et al. (2015b) (73%) and Elalfy et al. (2021) (73.1% in the cage and 68.8 % in the estuary) and BW had a negative significant effect on moisture (when fish weight increased 100g the moisture percentage decreased 0.7 %). This result was logical due to the high negative correlation between fat and moisture (García-Celdrán et al., 2015b; Vallecillos et al. 2021).

Table 2. Phenotypic results (least square means ± standard error) for body weight, fork length, and flesh composition for gilthead seabream.

		Offspring		Cov BW_{980dph}	
Traits	**n**	**mean**	**S.E.**	**b**	**S.E.**
BW_{251dph} (g)	392	43.7	0.95	NI	
TL_{251dph} (cm)	392	13.8	0.09	NI	
BW_{980dph} (g)	392	450.2	4.14	NI	
TL_{980dph} (cm)	392	28.7	0.11	NI	
Fat (%)	389	10.1	0.12	0.006*	0.002
Collagen (%)	388	1.74	0.02	0.001*	0.000
Moisture (%)	389	64.6	0.11	-0.007*	0.001
Protein (%)	389	19.8	0.06	<0.000	0.001

BW_{251dph} and BW_{980dph} = body weight at 251dph and 980 dph respectively; TL_{251dph} and TL_{980dph} = total length at 251dph and 980 dph respectively; * = covariate was significant ($P < 0.05$). NI: not included.

Gilthead seabream fillet showed the highest percentage of MUFA, followed by PUFA and SFA with similar percentages(Table 3). Fillet FA composition was closely related to the diet composition but not totally. In fact, in comparison with the diet, fillet showed higher SFA especially for 16:0, and lower MUFA and PUFA percentages, mainly due to the lower percentages of 18:1n9, 18:2n6 and 18:3, although for 22:6n3 the percentage increased notably in comparison to the diet. This difference in FA composition is largely explained by variations in the level of fattening, especially intramuscular fat, the percentage of PUFA, one of them DHA, decreased when FLC increased (Suomela et al., 2016). In our study, although the fish showed high FLC, the BW was much lighter than in Ballester-Lozano et al. (2011) and it is likely that they had not finished their development and fat deposition, and concurrently the DHA was high.

The main fillet FA were 18:1n9, 16:0, LA, and DHA, in accordance with that described by other authors (Ballester-Lozano et al., 2011; Fountoulaki et al., 2009; Sabbagh et al., 2019).

Table 3. Main fatty acids (%) of gilthead seabream flesh adjusted to 10.1 fat percentage.

FA	Offspring			Covariate fat percentage	
	n	LSM	S.E.	b	S.E.
14:0	394	3.29	0.03	0.011	0.015
16:0	371	17.9	0.23	0.018	0.090
18:0	392	4.86	0.11	-0.019	0.045
SFA	397	28.3	0.30	-0.002	0.130
16:1	397	0.32	0.04	-0.018	0.015
18:1n9c	392	33.6	0.70	0.232	0.277
18:1n9t	333	3.55	0.11	-0.005	0.047
20:1	392	2.38	0.03	-0.004	0.014
22:1	397	0.80	0.02	-0.014	0.008
MUFA	397	44.38	0.57	0.291	0.209
18:2n6	334	15.6	0.21	-0.054	0.084
18:3n3	389	3.77	0.05	0.055*	0.022
20:4n6	399	0.63	0.02	-0.017	0.006
20:5n3	381	2.88	0.04	0.008	0.016
22:6n3	384	6.84	0.12	-0.097*	0.048
PUFA	397	29.84	045	-0.400	0.165
∑ n6/ n3	394	1.05	0.35	0.249	0.140
UFA/SFA	392	2.97	0.08	-0.029	0.034

LSM: Least Square Mean, SE: standard error; SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids, UFA: unsaturated fatty acids; * = covariate was significant ($P < 0.05$).

2.3.2. Microsatellite Genotyping and parental assignment

The use of multiplex SMSa1 PCR using the exclusion method, with a maximum of two tolerated errors, provided successful parental assignment for 91.4 % of the offspring. After the assignment, six out of 76 females contributed with 52.1% of the offspring and 29 females did not produce any offspring, whilst six out of 57 males contributed with 60.9% of the offspring and 19 males did not contribute. Pedigree construction using selected highly informative microsatellite markers yielded 66 full-sib families with a mean of 3.86 sibs (range 2-28 sibs).

Regarding the study of genetic variation considering the microsatellites genotypes, high heterozygosity was observed, reaching 0.75. This value is consistent with the fact that the population came from a broodstock that had never been subjected to selection, and reveals that, at that moment, there was no danger of inbreeding.

2.3.3. Genetic parameters

- Heritability for growth traits

Heritability for BW_{251dph} and BW_{980dph} was moderate (PM=0.22 and HPD = [0.06-0.40]; PM=0.24 and HPD = [0.06-0.48] respectively). For TL, heritability was moderate (0.19 [0.04-0.36] at 251dph and high (0.48[0.18-0.80]) at 980dph (Figure 1), in accordance with other authors (García-Celdrán et al. 2015b; Ofori-Mensah et al. 2020; Sabbagh et al. 2019;)

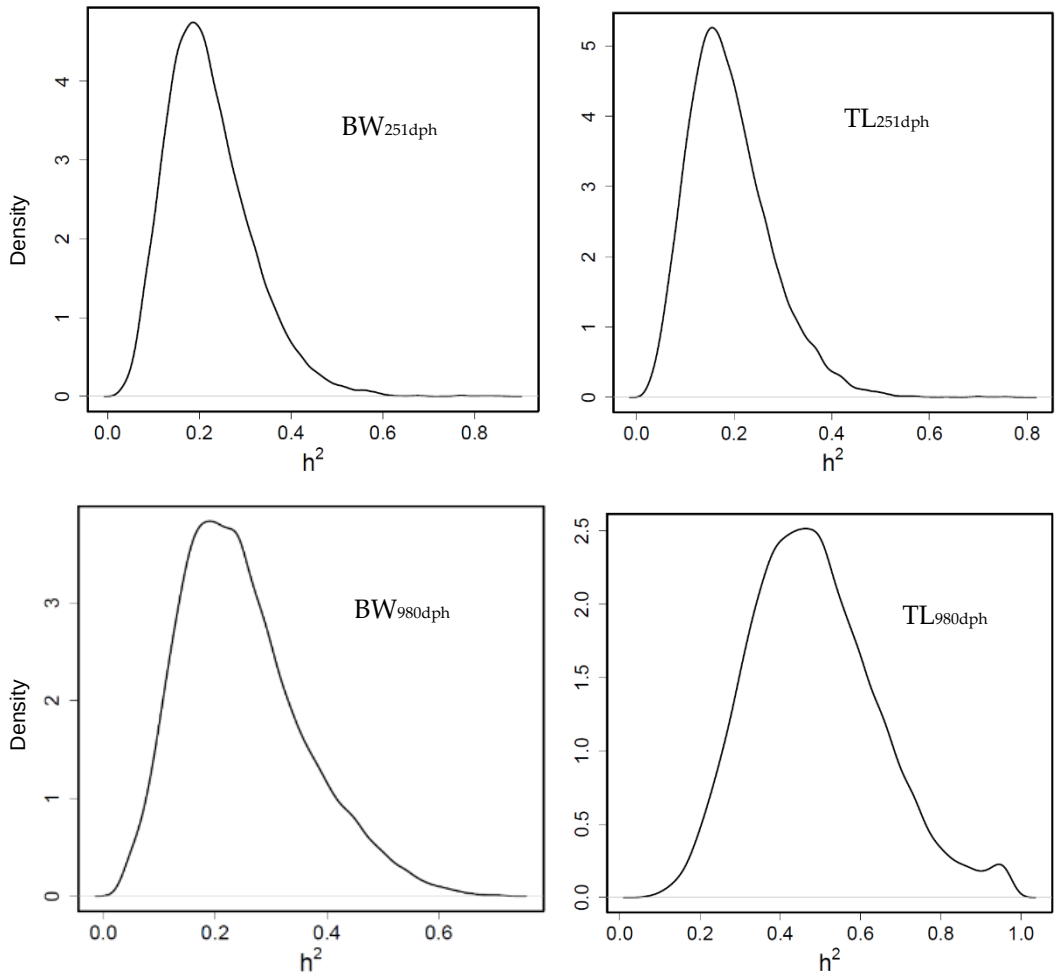

Figure 1. Posterior marginal distribution of heritabilities of body weight (BW) and total length (TL) gilthead seabream. h2=heritability (n= 399).

In our study, TL at advanced age was presented as more heritable than BW; however, other studies (García-Celdrán et al. 2015b; Ofori-Mensah et al. 2020; Sabbagh et al. 2019;) observed similar heritability for both traits and high genetic correlation between them, as also happened in our study (0.94±0.06 genetic correlation BW-TL_{980dph}). In addition, García-Celdrán et al. (2015b) pointed out that heritability estimates for growth traits increased with

age when they compared juveniles with commercial size fish. In our study, genetic correlation (rg) for BW or TL at different age were practically null but a safe interpretation of the rg is made difficult by the large standard errors (rg BW_{251dph}-BW_{980dph} = 0.13 ± 0.38, rg TL_{251dph}-TL_{980dph} = 0.04 ± 0.39).

- Heritability of flesh composition

Moderate heritability was obtained for fillet fat percentage (0.24 [0.06-0.44] PM and HPD in brackets, Figure 2) agree with Elalfy et al. (2021) and García-celdrán et al. (2015b), who showed 0.27 and 0.31, respectively. In our study, protein percentage heritability was moderate (0.21 [0.03-0.41]), however García-celdrán et al. (2015b) and Elalfy et al. (2021) reported low protein heritability (0.03 and 0.08, respectively). Regarding the collagen percentage, the heritability was low (0.06 [0.002-0.19]) in this study, similar to that in García-celdrán et al. (2015b) (0.03) and Navarro et al. (2009b) (0.02). The moisture percentage showed a medium genetic heritability (0.15 [0.015-0.32]) in the present investigation, however it has been reported with considerable variation between other studies ranging from medium to low heritabilities, such as Garcia-celdrán et al. (2015b) and Elalfy et al. (2021) (0.24 and 0.29, respectively) and Navarro et al. (2009b) (0.09).

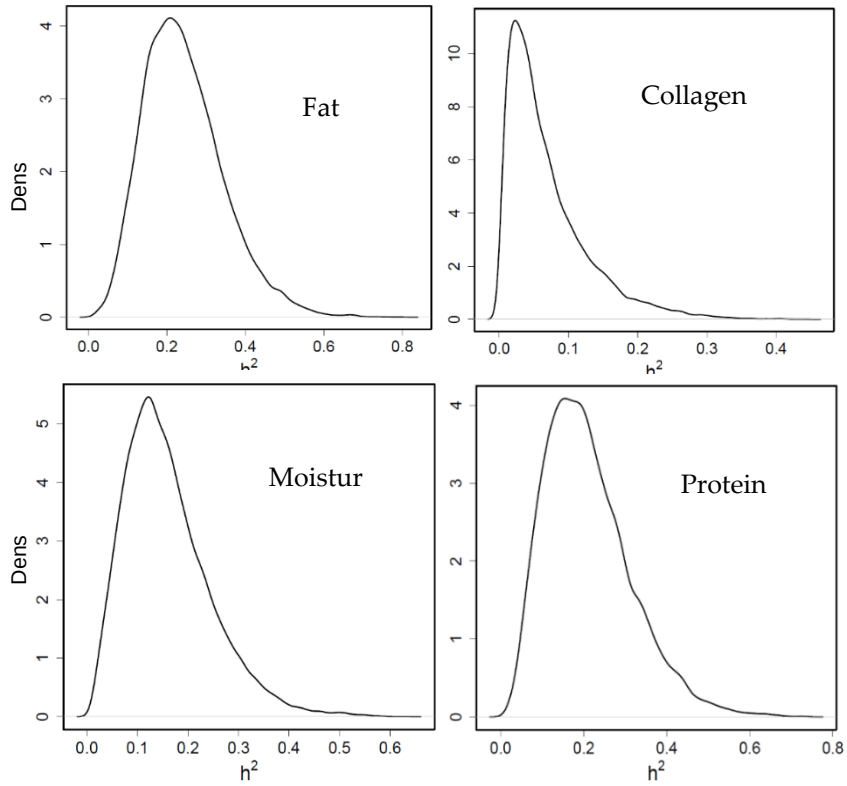

Figure 2. Posterior marginal distribution of heritabilities of fillet fat, collagen, moisture, and protein percentage in gilthead seabream. h2=heritability. (n= 399).

It is interesting to know the genetic correlation between BW and fat percentage, since most breeding programs select fish to improve their growth. In our study, the genetic correlation between both traits was not estimated with precision because of the limited data available. When this correlation was estimated (Elalfy et al. 2021; García-Celdrán et al.2015b;), a positive medium-high genetic correlation was observed, indicating that when fish are selected by growth, their fillet fat percentage increased indirectly.

- Heritability of fatty acid profile

Heritability was moderate for 14:0 (0.24 [0.04-0.48]), 16:0 (0.15 [0.01-0.33]) and 18:1n9c (0.20 [0.005-0.43]); it was low for 20:1 (0.12 [0.01-0.26]), and 22:6n3 (0.11 [0.017-0.25]); and practically null for 18:0 (0.02 [0.00-0.07]), 18:1n9t (0.03 [0.00-0.10]), 18:2n6t (0.09 [0.00-0.21]), 18:3n3 (0.05 [0.00 -0.15]) and 20:5n3 (0.05 [0.00-0.15]) (Figure 3) and 20:4n6 (0.03 [0.00-0.10]), 16:1 (0.04 [0.00-0.12]) and 22:1 (0.06 [0.00-0.16]) although the density plots for these last three FA are not represented.

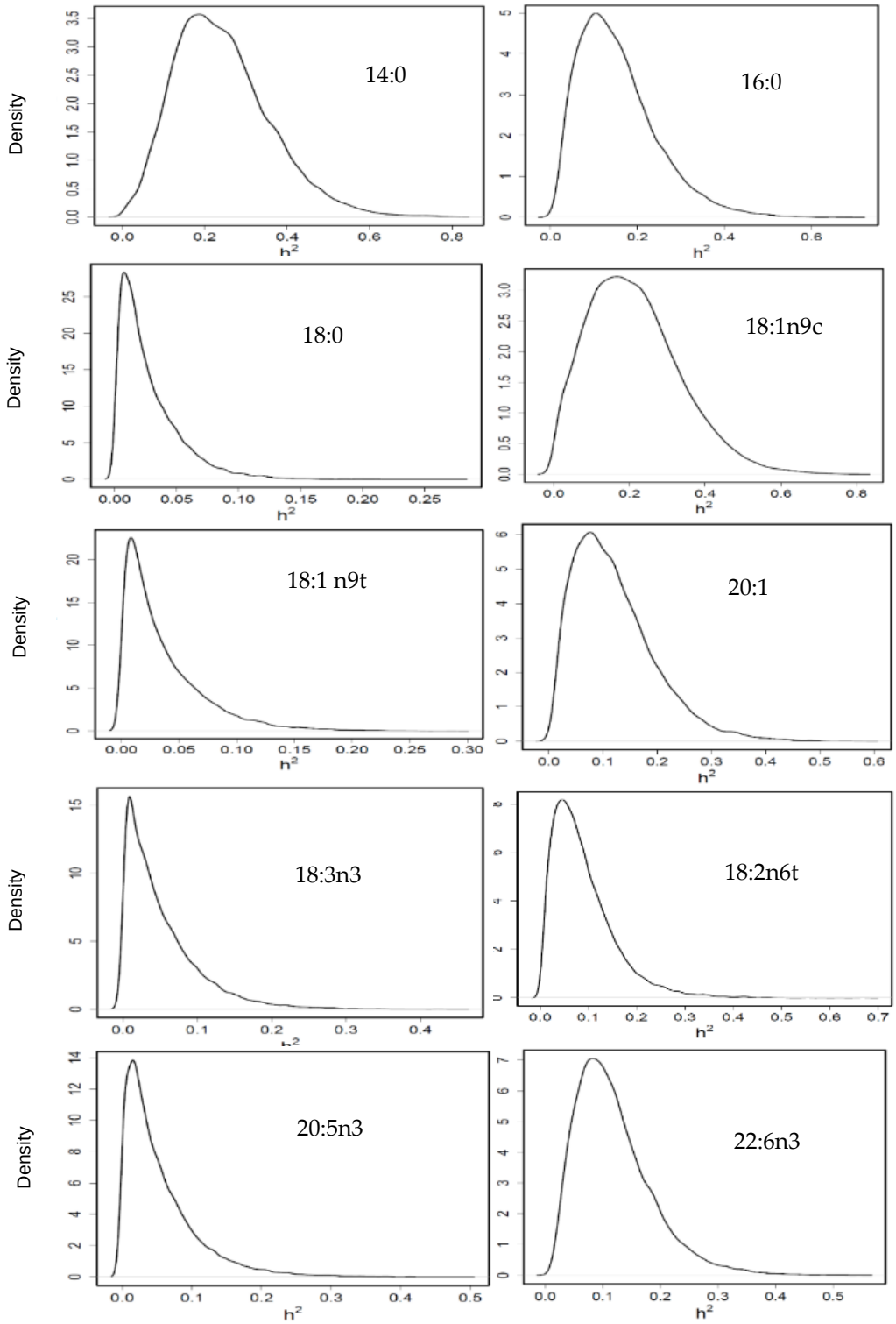

Figure 3. Posterior marginal distribution of heritabilities of fatty acids profile in gilthead seabream. h2=heritability. n = 399.

Heritability for the summatory of SFA (0.06 [0.00-0.17]) and PUFA (0.02 [0.00-0.09]) was almost zero; low for the ratio UFA/SFA (0.12 [0.01-0.26]); and medium for MUFA (0.26 [0.03-0.53]) and n6/n3 ratio (0.25 [0.03-0.49]) (Figure 4).

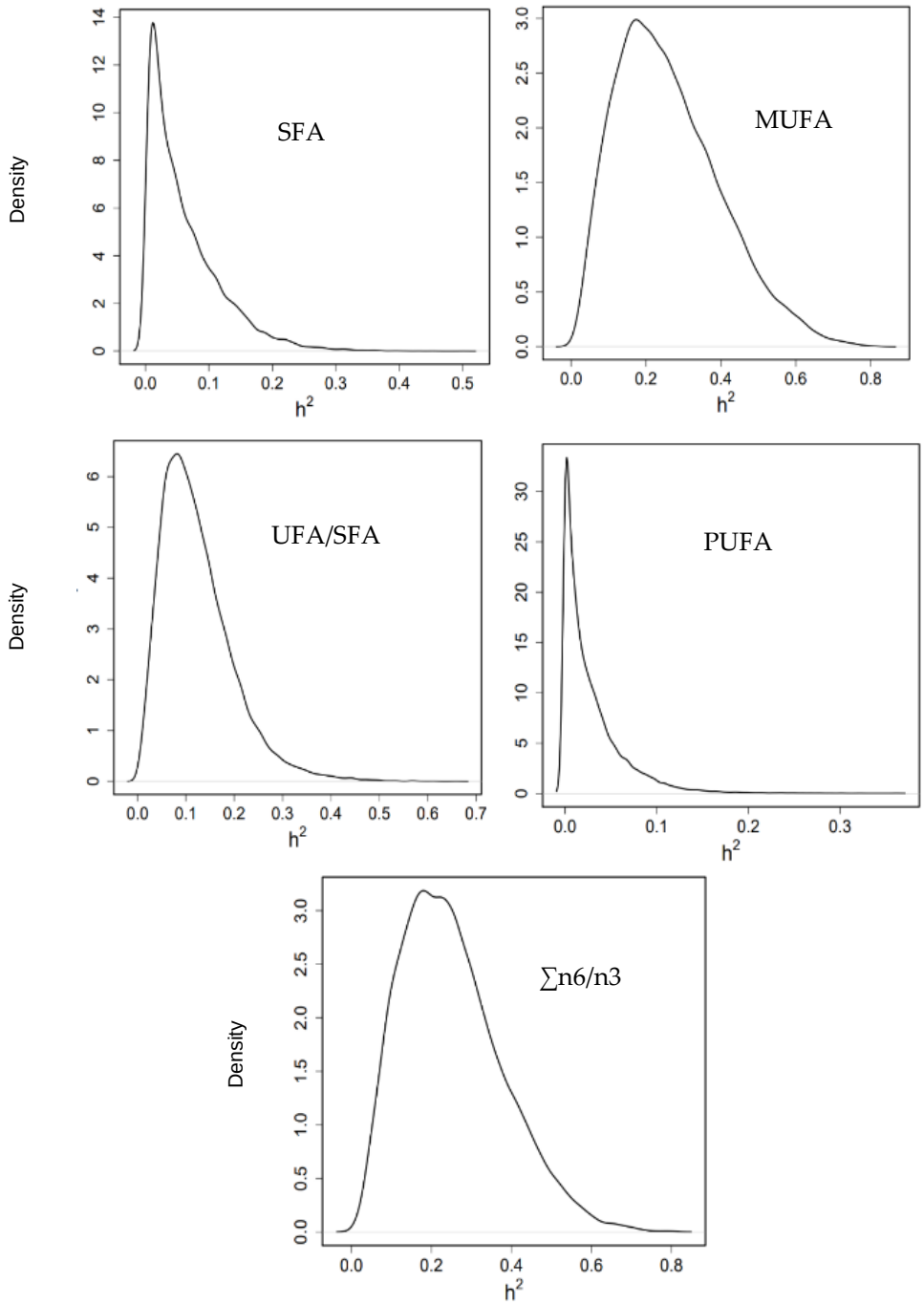

Figure 4. Posterior marginal distribution of heritabilities of SFA: saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, UFA: unsaturated fatty acids and the ratio omega 6/omega 3 fatty acids in gilthead seabream. h2=heritability. 399 data.

To our knowledge, there is no study about genetic parameters of FA in gilthead seabream. In Nile tilapia, the heritabilities for SFA were generally moderate, and for MUFA, PUFA and for total SFA, total MUFA, total PUFA, n3/n6 and UFA/ SFA were low (Nguyen et al., 2010). In Atlantic salmon, flesh n3 LCPUFA composition was highly heritable (h2 = 0.77±0.14) and the authors (Leaver et al., 2011) observed that families with a high percentage of n3 LCPUFA in flesh presented higher expression for genes related to hepatic lipid transport, and implicated increased activity of a transcription factor, hepatic nuclear factor 4α (HNF4α), possibly as a result of family differences in transforming growth factor b1 (Tgfb1) signalling. In that study, the authors (Leaver et al., 2011) also highlighted that FLC was highly and negatively correlated with percentage n3 LCPUFA (-0.77), and FLC was positively correlated to BW. In Nile tilapia (Nguyen et al., 2010), the genetic associations of the PUFA group (20:5n3 and C18:3n6) with BW traits were strongly negative (−0.55 to −0.78); and for two SFA the genetic correlations of 18:0 and 24:0 with fillet fat percentage were negative (−0.11 and −0.85, respectively). In our study, genetic correlations between FA and fillet fat percentage could not be estimated with precision, likely due to limited data availability. However, phenotypic correlation PUFA-Fillet fat percentage was significantly negative (-0.12). A major part of LCPUFA are in the membrane phospholipids (PL), which presents an upper threshold, because amounts of PL molecules in tissue are likely determined by a volume of membranes. Thus, when the level of fattening increases in a fish, most of that fat is deposited in muscles, as TAG, to be an energy reserve. Thus, when FLC increases, fat deposits increase, TAG content increases and PL, together LCPUFA, are diluted (Sushchik et al., 2020).

In shrimp (Nolasco-Alzaga et al., 2018), limited heritabilities for FA were estimated; nevertheless, some important FA, such as DHA had significant variance among families with similar heritability (0.12 ± 0.06) to our study. Also, in accordance with us, ARA, which is tightly linked to the immune response, showed a heritability not significantly different from zero.

Therefore, considering the positive genetic correlation between growth and fillet fat content, and the negative genetic correlation between fillet fat content and PUFA percentage, breeding for fish with higher growth is expected to cause an increase in the fillet fat percentage and a decrease of its PUFA percentage. In addition, most of the SFA and oleic, DHA, MUFA and the ratio n6/n3 have been shown to be heritable traits, thus their analyses should be considered in a breeding program.

The measurement of FA is expensive and time consuming, therefore further studies should be continued to investigate the relation between fillet fat content and its FA profile. The

Fish Fat Meter device (Distell.com) has been developed as a non-invasive tool to measure flesh fat content and a high correlation with FLC (Elalfy et al., 2021) has been demonstrated, thus it could be used as an easy non-invasive measurement.

2.4. Conclusions

Breeding programs in gilthead seabream usually include growth as the first criterion in the selection process. However, other quality traits, such as fillet fat percentage and its fatty acids profile should be considered, since they are very important traits for the consumer from a nutritional point of view. In addition, these quality traits are also related to the fish immune system and, consequently, to its disease resistance. Further studies to investigate the consequences of selecting fish for growth based on their fat content and their fatty acids profile are advisable.

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3.0. Abstract paper 3:

To carry out the study of genetic parameters in meagre (*Argyrosomus regius*), it was necessary to previously develop a parentage test using a microsatellite molecular marker panel, since none was available unlike in gilthead sea bream. Microsatellite panels are a power and very effective tool for parentage testing. The study was conducted with a population of meagre broodstock (9) and their offspring (616) from the Avramar S.L. group. Initially, 21 microsatellite markers were tested, but after testing them for poor amplification quality, low polymorphism, unclear banding pattern and intermediate alleles, we were left with a panel with eight meagre-specific and interspecific microsatellite markers, which was named Super Multiplex *Argyrosomus regius* (SMAr). The results of the 95% assignment to a single pair of possible parents, using the exclusion method implemented in Vitassing software (v.8_2.1), are shown.

Paper 3. Development of the first microsatellite multiplex PCR panel for meagre (*Argyrosomus regius*), a commercial aquaculture species

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Fishes 2022

Abstract

In this study, a microsatellite based multiplex PCR panel for meagre (*Argyrosomus regius*) was developed as a useful and single tool in parental assignment and population studies. 21 specific and interspecific microsatellites from different aquaculture species of meagre (*Argyrosomus regius*), Japanese meagre (*A. japonicus*), red drum (*Sciaenops ocellatus*) and yellow meagre (*Acoupa weakfish*) were assessed for genetic variability, allelic range and genotype reliability. Finally, a SuperMultiplex for *Argyrosomus regius* (SMAr) was designed with only the best eight microsatellite markers. The panel assessment was performed using a batch of broodstock from one company and a sample of 616 offspring. It was possible to assign 95% of the offspring to a single pair of parents using the exclusion method. It is therefore considered an easy procedure, and a power and low-cost tool for parental assignment to support companies breeding programs and to exchange information between research groups.

Keywords: Meagre (*Argyrosomus regius*); PCR; Microsatellites; Parental assignment; Population.

3.1. Introduction

Meagre (*Argyrosomus regius*) has been one of the most important emerging fish in the Mediterranean aquaculture diversification, with a production of 12,094 tons in 2019. The main meagre producing countries were Spain (4,505 tons), Turkey (3,375 tons), and Greece (2,415 tons) (FAO 2019).

As a consequence of the growth and consolidation of the production of meagre, the industries and production companies seek the implementation of improvements in the production cycles, in order to reduce production costs and obtain a better-quality final product. To achieve this, the implementation of a selective breeding program is essential. As in most fish, reproduction in meagre takes place through mass spawning, so the parents of the new individuals are not known, and this data is needed when estimating genetic parameters (Cárdenas, 2010) (p. 32). Fish breeding programs are based on the identification and tracking of the individuals both through physical tagging as well as the genetics of parent–offspring relationships by analyzing molecular markers to avoid expensive genetic analysis at all sampling points during the on-growing process (Lee-Montero et al., 2013). Different genetic markers are used to investigate the relationship between relatives and to assist selection

programs. Genome-wide distributed molecular markers, such as Single Nucleotide Polymorphism (SNPs) are useful in both parental assignment with relatively few markers (150-200) and in genomic selection with a large number of markers (>30K), where they are found to improve the accuracy in estimating breeding values (Aslam et al., 2018; Palaiokostas et al., 2016); and microsatellites (Lee-Montero et al., 2013), which have been widely used in parental assignment, gene mapping and population genetics studies (Lee-Montero et al., 2013; Ma et al., 2021). A selection scheme requires designing a standardized system with a good allocation of family relationships. Different variables such as population size and allelic diversity (Villanueva et al., 2002), the number of breeders to be used in spawning (Liu & Cordes, 2004), the level of homozygosity (Marshall et al., 1998) and the existence of null alleles (Lee-Montero et al., 2013) must be studied when selecting the necessary number of loci to obtain a successful assignment.

A panel of genetic markers is a set of highly polymorphic, specific and reproducible markers, assessed according to their polymorphism and their genotyping errors. Microsatellites are used as molecular markers in a panel. When combining microsatellites in a multiplex PCR it reduces the cost, the execution times, the PCR errors, and produces good efficiency in the parental assignment (Lee-Montero et al., 2013; Villanueva et al., 2002). In the development of a standardized panel, it is intended to facilitate the exchange of information, the combination of data sets and the management of populations between research groups. An international panel is that which is approved and recommended by the International Society for Animal Genetics (ISAG).

In the family of the Scianids, many microsatellites have been described: (Archangi et al., 2009; Chang et al., 2009; Farias et al., 2006; Li et al., 2008; Porta et al., 2010; Saillant et al., 2004; Turner et al., 1998). To date, in the case of meagre (*Argyrosomus regius*) no microsatellite markers panel has been developed, although several specific and interspecific microsatellites have been used in several works (Archangi et al., 2009; Farias et al., 2006; Kathleen et al., 2003; Porta et al., 2010; Saillant et al., 2004), and only a few studies (Nousias et al., 2020, 2021; Soula et al., 2012) showed the multiplex they used for the parental assignment. Vallecillos et al. (Vallecillos et al., 2021) developed a panel of markers similar to this study but with some minor modifications. Several multiplex PCRs for parental assignment have been described for gilthead sea bream (Borrell et al., 2011; Launey et al., 2003; Navarro et al., 2008) for long-snouted seahorse (López et al., 2012), and recently for white-leg shrimp (Ren et al., 2022), and for Crassostrea hongkongensis (Ma et al., 2021).

The main objective of the present study was to develop a microsatellite based multiplex PCR panel to homogenize procedures, to enable results to be compared and facilitate the exchange of information between laboratories, and to provide a useful genetic tool to facilitate future selective breeding in aquaculture industry.

3.2. Materials and Methods

3.2.1. Sample

Six hundred sixteen offspring from a broodstock of nine breeders (4 males and 5 females) from Alevines del Sureste SL company were used to assess the quality of a microsatellite based multiplex PCR panel in the parental assignment.

DNA was extracted from the caudal fin, which was preserved in ethanol, using the DNeasy kit (QIAGEN®), and stored in a freezer (-20 ºC) until the next process. Next, DNA quantity and quality were determined with a NanoDrop™ 2000 spectrophotometer v.3.7 (Thermo Fisher Scientific, Wilmington, U.S.A.). The integrity of the DNA was verified by 1% agarose gel electrophoresis staining with ethidium bromide and analyzed by the photo documentation kit (AlphaDigiDoc RT2), using DNAMARKER Beethoven (Danagen-Bioted, Spain) as molecular weight marker.

3.2.2. Primer desing

From the microsatellites published in the genetic map of different species of Sciaenidae, 11 microsatellites were markers of meagre, *Argyrosomus regius* (Porta et al., 2010); six of Japanese meagre, *A. japonicus* (Archangi et al., 2009); three of red drum, *Sciaenops ocellatus* (Kathleen et al., 2003; Turner et al., 1998); and one of yellow meagre, *Acoupa weakfish* (Farias et al., 2006), so we had 21 pairs of primers which were used to amplify microsatellites using the same PCR conditions (Table 1). The length of the amplicons ranged between 80-266 base pairs (bp), thereby ensuring a wide amplification spectrum, and optimizing the efficiency of the multiplex reaction (Dakin and Avise, 2004). To avoid complexity problems, a theoretical annealing temperature of 60 ºC ± 2 ºC was sought for all primers.

Table 1. Microsatellites tested for panel development in meagre (*Argyrosomus regius*).

Authors	Species	Loci
Porta et al. (2010)	*Argyrosomus regius*	gCT15, gA2B, CA3, CA4, CA6, CA14, CA13, CA10, gA16, gA17 and gA6
Archangi et al. (2009)	*Argyrosomus japonicus*	UBA853, UBA54, UBA53, UBA50, UBA6 and UBA5
Turner et al. (1998)	*Sciaenops ocellatus*	Soc 011
Kathleen et al. (2003)	*Sciaenops ocellatus*	Soc 431 and Soc 405,
Farias et al. (2006)	*Cynoscion acoupa*	CacMic 14

3.2.3. PCR conditions

Each microsatellite was individually tested using a PCR with part of the samples (12% of the sample), in order to test and confirm its correct amplification, allele size range, genetic variability and genotyping. The PCR was performed in a 12.5 µl reaction mix the following component concentrations: 2X Type-it Microsatellites PCR kit (QIAGEN®, Hilden, Germany), 10 µmol/l for each primer and 10 ng/µl template DNA. The thermal profile included a pre-denaturation step of 95 ºC for 15 min followed by 32 cycles of denaturation-annealing-extension at 94 ºC for 30 s, 60 ºC for 90 s and 72 ºC for 1 min, and one final elongation step at 60 ºC for 30 min. Later, 1 µl of each reaction product was mixed with 6.32 µl of Hi-Di formamide and 0.36 µl of GeneScan LIZ 500 (Applied Biosystems) and amplicons were resolved by capillary electrophoresis on an ABI 3730 sequencer (Applied Biosystem, Foster City, CA, U.S.A.). The fragment size analysis software ThermoFisher MSA was used for genotyping.

3.2.4. Genotyping reliability of microsatellite markers

Each microsatellite marker was assessed individually, with four quality controls being considered (Lee-Montero et al., 2013): a) inadequate amplification: peak height <300 RFU (relative fluorescent units); b) null allele: preferential amplification of the shortest allele; c) unclear band pattern: bands that make it difficult to identify between homozygous and heterozygous for adjacent alleles; d) intermediate alleles: loci of di-nucleotides, differ from each other by 1 bp (Figure 1). The frequency of null alleles was estimated using MicroChecker software (2.2.3) (van Oosterhout et al., 2004) which estimated allele frequency using four different algorithms.

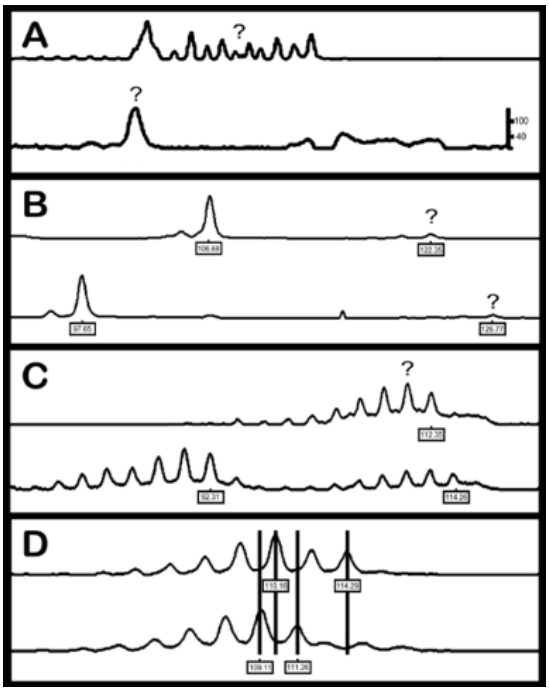

Figure 1. Potential errors during genotyping reliability of microsatellite markers. a) inadequate amplification, b) null allele, c) unclear band pattern, and d) intermediate alleles (source: (Lee-Montero et al., 2013))

The concentration of the primers was adjusted until peaks between 600 and 3000 RFU were obtained, as described by Navarro et al. (2008). The PCR product was again checked on a 2% agarose gel for 45 minutes to test the amplification of the multiplex amplicons. As before, the amplification reading of each locus was performed under the same conditions used in the individual PCR. The electrophoregrams were analyzed by ThermoFisher MSA.

3.2.5. Genetic diversity and parental assignment

Using the Cervus package (3.0.7) (Kalinowski et al., 2007), the Hardy-Weinberg (HW) equilibrium test, the observed heterozygosity (Ho), expected heterozygosity (He) and the polymorphic information content (PIC) were studied. The markers were classified according to their PIC value as being highly informative (PIC ≥ 0.5), reasonably informative (0.5 > PIC > 0.25), and slightly informative (PIC ≤ 0.25) (Lee-Montero et al., 2013). Wright's Fixation index (FIS) was calculated as (Mean He - Mean Ho) / Mean He with the GenAlex software (v.6.0.) (Peakall and Smouse, 2012), indicating this coefficient departure from HW expectation. For the parental assignment, the exclusion method as implemented in VITASSING (8.2.1) software (Vandeputte et al., 2006) was used. Breeders effective size (Ne) was firstly estimated as NêF = (4NsNd)/(Ns + Nd) (Reeve, 1989) where Ns is the number of sire breeders and Nd the number

of dam breeders. We also used an approach that accounts for variance in family size: NêC = 4(N - 2)/((Ks + Vs/Ks) + (Kd + (Vd/Kd) - 2)) (Crow and Denniston, 1988) with N being the offspring sample size, Ks and Kd the mean numbers of offspring per sire and per dam, and Vs and Vd the variances of sire and dam family sizes. It is a useful approximation to the problem of estimating Ne when there are unequal contributions of breeders to offspring.

3.3. Results and discussion

3.3.1. Microsatellite selection, multiplex PCR design and parental assignment

It is very important to perform the assessment of microsatellite markers one by one, so we did so according to the four potential errors (Lee-Montero et al., 2013). Miquel et al. (2006) reported that the reliability of genotyping is as important as the degree of polymorphism. In our study, the main problem was in the low or non-existent amplification, eight (CA6, UBA5, CA4, UBA853, CA14, UBA6, UBA54, and CA10) of the 21 microsatellite markers (38%) did not amplify; four were from *A. japonicus* and four from *A. regius*; this problem with the amplification was probably because some microsatellite markers were interspecific and/or primers design problems. Two markers had inadequate amplification (CA13 and GA16) and they could not be well observed. In addition, the production of null alleles may reduce the success of the assignment, as seen by other authors (Marshall et al., 1998), for us two microsatellite markers showed a high rate of null alleles (21.1 and 24.3% null allele frequency for gCT15 and CA3 respectively, according to Oosterhout's estimation). Finally, the less frequent error was the appearance of intermediate alleles, thus only one microsatellite showed intermediate alleles (GA6), however in Lee-Montero et al. (2013), this error was the most frequent (16%).

The remaining eight microsatellites were selected as suitable (Table 2). A multiplex PCR of only these eight microsatellite markers was developed to amplify all of them in the same reaction. For this purpose, the amplicons size, different fluorochrome, the observed allele size range were considered, and the concentration for each microsatellite marker optimized after several genotyping runs (table 2), also the allele frequency for the microsatellite marker was studied and is shown in the supplementary table S1. In this study, the amplification size was similar in all markers (from 80 to 155 bp), except in SOC011 which has a larger size than the rest, but its amplification was appropriate. The minimum distance between markers with the same fluorochrome after genotyping was 12 bp, avoiding overlaps between them. In the process

of unifying different microsatellite markers in the same PCR, it is recommended, in addition to starting from a good quality of DNA, to select those that have a similar size so as not to reduce the amplification of some loci (Dakin and Avise, 2004). However, other authors opted for microsatellites with different amplification sizes (Borrell et al., 2011; Navarro et al., 2008; Porta et al., 2010), to avoid overlaps between microsatellite markers dyed by the same fluorochrome.

Table 2. Microsatellite panel of Meagre: GenBank accession number, motif, the tagging fluorochrome, the primers sequence, concentration and allele size range.

STR loci	GenBank Accession number	M	F	Forward sequence (5´-3´)	Reverse sequence (5´-3´)	Concentration (µM)	Allele size range
gA2B	GU724794	$(CA)^{26}$	5*NED	AAGTGTGGCGTCATTTCCTCT	GTATTGATGGATAGCAAGTGTCAGA	0.06	86-110
UBA50	EF462924	$(GT)^{26}$	5*NED	GCACAACTGCATCCCTTAGAT	GTTTAGAAGTGAAGACTGCGGACTG	0.15	128-152
gA17	GU724798	$(GT)^{12}$	5*6-FAM	CTAGAGAAATTCATCCAGGGAAGTG	GTTTAGAGCAGAGAGTTAGCGGTTGTT	0.06	80-92
SOC 405	AY161014	$(CA)^{12}$	5*6-FAM	AGCCTTTTGTTTAGTTTCCCTCAT	GGGGTGTAGCAGAACCACAC	0.06	112-124
Cacmic 14	DQ285034	$(CT)_{12}$	5*PET	ATCTTCTCCCCTCCGTCACT	CTGTGTTGTTAAGGCGCATC	0.06	132-148
SOC 431	AY161032	$(GT)^{26}$	5*PET	GTGGTAGATGAAAACGTATAAAAGGAG	GTTTCATATATATAGTGTACAGCTCCAGCTTC	0.08	124-148
UBA53	EF462925	$(CA)^{14}$	5*VIC	TACTTCCTTCTACCCCTAAGTCTGG	GACTTTCCAGTGTAGCTGTCGTTT	0.08	86-114
Soc 011	AF073258	$(GA)_{11}$	5*VIC	GCCGAGTCACGAAGGAACAGAGAA	TGTCGTCTCATCTATCTCCATCTC	0.08	250-266

M: motif of repetition; F: fluorescent tag.

In the present study, only eight microsatellite markers formed our standardized panel and were combined in an abridged SuperMultiplex for *Argyrosomus regius*, which was named "SMAr". Parental assignment was carried out with SMAr allowing for four mismatches at first reading of genotypes, with no prior correction for mismatches, but nevertheless, most of the offspring (64.7%) were assigned with one or two mismatches to a single pair of parents (Table 3). The power of SMAr is that it allows us successfully assign 95.1% of offspring to a single pair of parents at the first genotyping reading. All the parents contributed to the spawning, although unequal contribution was observed, two out of five females produced 61.8% of the offspring although all the females contributed to the offspring, and one out of four males contributed with 49.7% of the offspring and all the males contributed. The effective breeding number estimating considering only contributing males and females ($N\hat{e}_C$ = 6.9) were lower than the former ($N\hat{e}_F$ = 8.9) assuming of breeders spawned. Therefore, a first reduction in the effective breeding number was observed. There are many authors that develop a panel of microsatellite markers for the management of the pedigree of the study populations and to support breeding programs (Lee-Montero et al., 2013; López et al., 2012; Ren et al., 2022). Nousias et al. (2020) for meagre with 10 microsatellites panel reached an overall 91.1% parental assignment, Lee-Montero et al. (2013) for gilthead seabream used two panels with 11 markers in each (SMsa1 and SMsa2) with 100% of assignment, Vandeputte et al. (2006) for rainbow trout with 5 microsatellites panel showed 57.1% assignment to a single correct and 40.3% to multiple pair of parents at the first reading of genotypes, using the same VITASSING software. Ren et al. (2022) for white-leg shrimp used two panels with 6 markers in each (SMpv01 and SMpv02) that were tested using CERVUS and COLONY with a 100 and 94.3% of assignment and López et al. (2012) for long-snouted seahorse used a panel with 6 markers, that were tested using CERVUS and FAP with 100 and 95.8% assignment. Previous studies did not point out the number of reading of genotypes, therefore we suppose that the shown parental assignment percentage is after genotype corrections. Only, Vandeputte et al. (2006) highlighted that the assignment was at the first reading of genotype.

Table 3. Number of offspring assignment from parental pairs identified with eight microsatellites when four mismatches are allowed, in 616 fish.

	Maximum number of mismatches allowed				
Kind of assignment observed	**0**	**1**	**2**	**3**	**4**
Single pair of parents	83	157	159	146	41
Multiple pair of parents	0	5	7	1	2

2.4% (15 offspring) of the evaluated offspring were not assigned to any possible pair of parents.

3.3.2. Genetic diversity

Genetic diversity, analyzed by heterozygosity and polymorphism degree of each microsatellite, FIS and HW equilibrium is shown in Table 4. In our population of offspring, for the selected microsatellite markers the mean values and the range were: for number of alleles 7.9 (from 5 to 13), for observed heterozygosity (Ho) 0.69 (from 0.35 to 0.97), for expected heterozygosity (He) 0.61 (from 0.32 to 0.81), for polymorphism information content (PIC) 0.57 (from 0.35 to 0.80) and for FIS -0.12 (from 0.03 to -0.22).

In our population, mean Ho was high, and mean He was slightly lower for most microsatellites; except to SOC405 and UBA53, which showed a lower Ho because one or two allele showed a much higher frequency than the others (Table S1). Other studies have observed slightly less heterozygosity (Soula et al., 2008, pp. 21-24) than in our study, which is probably due to the fact that we have chosen highly polymorphic microsatellites and, although the number of breeders was very small in our work, all of them contributed and have never been subjected to a selection process. In other aquaculture species such as gilthead sea bream something similar occurs, since they are stocks that have not undergone any breeding program (Lee-Montero et al., 2013; Navarro et al., 2008), observing a high population heterozygosity. Most of the microsatellites showed a significant HW disequilibrium, which revealed an excess of heterozygotes, maybe due to the small effective number of breeders and their unequal contribution (García-Celdrán et al., 2016), as it is reinforced with the lower $N\hat{e}_C$ estimated when we applied the Crow and Denniston (Crow and Denniston, 1988) approach considering the true variance family size.

A microsatellite based multiplex PCR panel have to be sufficiently informative to ensure high quality results (Selkoe and Toonen, 2006). In our work, five out of eight microsatellites were highly informative and three were reasonably informative (gA2B, SOC405 and UBA53), as similar result happened in the multiplex proposed by Lee-Montero et al. (2013), although the level of polymorphism of the microsatellite markers also depends on the population (Vandeputte et al., 2011). In addition, the use of non-coding regions in markers favors that their genetic variability is not lost over time, especially when they are used in breeding programs and some selection pressure is exerted on them (Lee-Montero et al. 2013), so mutation and drift are the main factors driving allele frequency changes within and among populations, although draft due to close linkage to coding regions has been suggested (Hansen et al., 2007). For coding markers, Chistiakov et al. (2006) observed how reduced their variability in successive selected generations.

Table 4. Genetic diversity of the offspring population using the optimized Super Multiplex *Argyrosomus regius* (SMAr).

Meagre STR loci	**Nº alleles**	**Ho**	**He**	**PIC**	F_{IS}	***P*-value HW**
gA2B	8	0.62	0.52	0.46	-0.19	***
UBA50	13	0.97	0.81	0.80	-0.21	***
gA17	7	0.73	0.69	0.68	-0.07	***
SOC 405	5	0.35	0.32	0.35	-0.10	***
Cac mic 14	8	0.79	0.72	0.70	-0.09	***
SOC 431	8	0.61	0.50	0.57	-0.22	***
UBA53	9	0.58	0.60	0.47	0.03	NS
Soc 011	9	0.88	0.80	0.77	-0.10	***

Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphism information content; F_{IS}: fixation index; HW: Hardy-Weinberg equilibrium; *** *P*-value ≤ 0.001 that implies disequilibrium HW. NS: not significant.

Future selective breeding for traits associated with aquaculture production efficiency is greatly desired in the aquaculture industry. However, one of the main concerns in a selection process is the remarkably reduced number of the effective population size that can lead to an increase in inbreeding. Indeed, we observed significantly unequal breeder contributions to the offspring. This outcome seriously affected the $\hat{N}_C$ estimates which decreased compared with

$N\hat{e}_F$, therefore inbreeding is likely to increase in subsequent generations. Microsatellite markers can help us to avoid such inbreeding matings.

A microsatellite based multiplex PCR panel is a useful and low-cost tool to enhance genetic breeding programs, through the stock management, the individual genetic identification, and pedigree reconstruction, and in population genetics studies. In this case, the panel has been designed to support and carry out the first genetic improvement program in meagre (GENECOR 2020) in Spain. The application of SNPs genetic markers to breeding is particularly valuable for difficult traits or that cannot be measured on the selection candidates (e.g., disease resistance and fillet quality) in which the accuracy of the estimated breeding values can improve (Aslam et al., 2018; Palaiokostas et al., 2016) However, for other traits associated with aquaculture production with medium-high heritability, such as growth (Vallecillos et al., 2021), the accuracy for estimated breeding values using a pedigree-based approach or genomic prediction is expected to be similar (Blasco and Toro, 2014). Furthermore, despite of the fact the cost of SNPs genotyping has been dramatically reduced in the last few years, the cost of genotyping with microsatellites (8-10 markers) has been estimated to be two to three times cheaper compared to SNPs (150-200 markers) when are used for parental assignments, 3 € for microsatellites versus 7-11 € for SNPs, not including DNA extraction.

3.4. Conclusions

The design of microsatellite markers SuperMultiplex for Argyrosomus regius (SMAr) as a panel, that amplifies all loci in one reaction, is a way to standardize procedures, and to share results among researchers and companies. The use of this panel is a power and economic tool in the parental assignment and to support a breeding program of greatly interest for aquaculture industry.

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3.6. Ethical approval

The animal study protocol was approved by the Ethics Committee Polytechnic University of Cartagena of the Region of Murcia (protocol code CEI21_006 and May 27, 2021)

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4.0. Abstract paper 4:

Meagre is an emergent species in aquaculture, due to its fast growth rate, low feed conversion ratio, and the high quality of the product. Although advances have been achieved in its management, reproduction, and feeding, breeding programs have not yet been developed. For this reason, this study aimed to provide information about the genetic variations in growth, morphology traits, and flesh chemical composition to be included in a selective breeding program. The study in meagre focused on the analysis of characteristics of commercial interest, such as growth variables (weight and length), morphological characteristics (standard length and height at the midpoint and peduncle of the caudal fin) obtained by image analysis, and meat quality (fat, protein, moisture, and collagen content). The study was carried out on a population of 616 meagre from a batch of broodstock from the company Alevines del Sureste, belonging to the Avramar S.L. group, which were reared in two different environments (cage and tank). The results showed that the fish reared in the cage presented higher values of growth and morphological characteristics and a higher percentage of fat, while the percentage of protein was lower than in the fish in the tank. In addition, mean heritabilities were obtained for growth characteristics, morphological characteristics and fat percentage, indicating that these are criteria that can be included in a genetic improvement program to obtain improved individuals for these variables. Image analysis proved very useful for evaluating growth and morphological variables, providing us with a large amount of information and more objective values, since this avoided possible errors in the case of manual measurements.

Paper 4. Phenotypic and Genetic Components for Growth, Morphology and Flesh-Quality Traits of Meagre (*Argyrosomus regius*) Reared in Tank and Sea Cage.

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Simple Summary: Meagre is an emergent species in aquaculture, due to its fast growth rate, low feed conversion ratio, and the high quality of the product. Although advances have been achieved in its management, reproduction, and feeding, breeding programs have not yet been developed. For this reason, this study aimed to provide information about the genetic variations in growth, morphology traits, and flesh chemical composition to be included in a selective breeding program, studied in two different housing systems (cage and tank). Heritabilities for growth and morphology traits, and for fillet fat percentage were medium, revealing those traits as a possible selection criterion in a breeding program. Image analysis provided a great amount of objective information regarding the different morphological traits of the fish, where a positive and high correlation with growth traits was observed. Positive phenotypic correlation between fillet fat percentage and body weight was observed, so a selection process to improve growth rate could lead to a fish with higher fillet fat percentage.

Abstract: Meagre (*Argyrosomus regius*) plays an important role in the aquaculture system, with the potential for diversification of European aquaculture, and is characterized by its fast growth rate, low feed conversion ratio, and the high quality of the product. Focusing on the relevance of meagre, the aim of the study was to analyze growth performance, fish morphology, and flesh composition phenotypically and genetically to be considered as a strategy in a breeding program. For this purpose, 633 fish were raised in two different housing systems, in sea cages or in a continental tank, and when they reached harvest size, manual growth traits, automatic morphology by the image analysis program IMAFISH_ML, and flesh chemical composition (fat, protein, moisture, and collagen percentages) were measured. The fish reared in the cages showed a higher body weight and fillet fat percentage than those in the tank. Heritabilities for growth and morphology traits, and for fillet fat percentage were medium, revealing these traits as a possible selection criterion in a breeding program. Phenotypic and genetic correlations between growth and morphology traits were positive and high. Phenotypic correlations between growth or morphology traits with fillet fat percentage were positive and medium; genetic correlations were not estimated accurately.

Keywords: Meagre (*Argyrosomus regius*); Infrared Spectroscopy (NIR); Moisture; Fat content; Protein; Collagen; Heritabilities; Correlations; Stock density.

4.1. Introduction

The meagre (*Argyrosomus regius*) is one of the most important fish in the diversification of Mediterranean aquaculture (DIVERSIFY, 2018). It is a teleost fish of the Sciaenidae family which can be found along the Atlantic coast of Europe, in the Mediterranean Sea and Black Sea, and the east coast of Africa, at depths of between 15 and 200 m (Cárdenas, 2010). It is also found in estuaries and coastal lakes (Griffiths and Heemstra, 1995). Meagre has an important role in the aquaculture system, as a potential species for diversification of European aquaculture, with its annual production in the Mediterranean area exceeding 41,000 tons (APROMAR, 2020). This species is easy to adapt to captivity due to its tolerance to a wide range of salinity (5–39%) and temperature (13–28 °C) (Cárdenas, 2010). The main interest in this species lies in its fast growth rate (around 800–1000 grams in 18 months) with a low feed conversion ratio (Fountoulaki et al., 2017). In addition, meagre have an attractive body shape as a whole fish commodity, a low-fat fillet content, and a good processing yield (La et al., 2006). Moreover, meagre has been proven, along with other aquaculture species, to be a great source of unsaturated fatty acids (Alexi et al., 2019; Poli et al., 2003; Saavedra et al., 2017), which are highly appreciated in human nutrition for their great benefits in several cardiovascular diseases (Augustsson et al., 2003; Mccullough et al., 2002).

Strategies need to be established in the different areas of knowledge to promote the consolidation of the meagre industry. Companies and research already support different actions in the field of feeding, with studies that have tested different diets, with different protein percentages or including new feeds (Estévez et al., 2022; Fountoulaki et al., 2017; Lozano et al., 2017; Piccolo et al., 2008; Poli et al., 2003), or reproduction and batch management, in which different reproduction techniques have been studied and how stocking density affects the final product (Duncan et al., 2012, 2018; Fakriadis et al., 2020; Piccolo et al., 2008; Poli et al., 2003; Schiavone et al., 2012; Vallés and Estévez, 2013). Most studies into disease prevention have focused on granulomas in all soft tissues (Andree et al., 2015; Carvalho et al., 2019; Elkesh et al., 2013; Tsertou et al., 2018).

However, strategies in aquaculture breeding programs are scarce, and particularly in an emerging species such as the meagre. Selective breeding programs are one of the fundamental strategies that must be included in any production system. In the case of terrestrial livestock, the accumulative annual genetic gain rate is estimated to be around 1–3% (López-Fanjul and Toro, 2007). The different programs in Europe are currently considering different objectives, such as growth performance and feed efficiency, morphology, product quality, processing yield,

reproduction, and disease resistance (Janssen et al., 2017). The selection criterion greatly affects the genetic progress through differences in additive genetic variation, which is essential for improving breeding values (Chavanne et al., 2016). For that reason, it is important to know about the genetic variation for the traits of interest in fish farming.

Thus, growth and feed conversion stand out as one of the main objectives in breeding programs, due to the high feeding costs. Due to the difficulty in measuring feed consumption, feed conversion is normally improved indirectly through growth traits such as weight and length (Navarro et al., 2009).

Regarding fish quality, the external appearance is one of the most important traits as irreversible modifications of morphology produce alterations with respect to the quality standard. In this case, several deformities have been described in meagre, such as granulomas (Carvalho et al., 2019); this can lead to reduced growth performance and fish that are visually unacceptable for the market.

To assess external morphology, Navarro et al. (2016) developed a non-invasive methodology that consists of a fast and automated software for image analysis (IMAFISH_ML), measuring 27 fish morphometric traits (technological traits) on three commercial fish species—gilthead seabream (*Sparus aurata* L.), meagre (*Argyrosomus regius*), and red porgy (*Pagrus pagrus*). Methodological tools, based on image analysis, have also been developed for the on-line sorting of farmed seabass (*Dicentrarchus labrax*, L.) by size, sex, and the presence of abnormalities (Costa et al., 2013).

To the best of our knowledge, only one work (Nousias et al., 2020) has studied genetic variation for growth traits in meagre, albeit only for body weight and length, whilst no study has considered the genetic component for other traits related to external morphology.

In other aquaculture species, other breeding objectives such as flesh quality and disease resistance have been established later (Janssen et al., 2017; Massault et al., 2011; Palaiokostas et al., 2016; Perera et al., 2019; Vallecillos et al., 2021). In meagre, there are no such studies of genetic variation for flesh composition and disease resistance, although the expression of the immune system genes has been analyzed (Campoverde et al., 2019).

The aim of the present study was to estimate genetic parameters of the main traits that are of fish farming interest, such as growth performance, morphology, and flesh composition, for the first time in meagre (*Argyrosomus regius*), to be considered as a strategy in a selective breeding program.

4.2. Materials and Methods

4.2.1. Ethics statement

To ensure that animal welfare standards were maintained, anesthetic was used in the sampling procedure. All animal experiments described in this manuscript fully complied with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council Universidad Politécnica de Cartagena of Región de Murcia, Spain (approval number CEI21_006).

4.2.2. Animals

The experiment was carried out with meagre, which were obtained from one broodstock (n = 9; 4 males and 5 females), belonging to the company Alevines del Sureste S.L. of the Avramar group. The broodstock were under a controlled photoperiod (8L:16D) to synchronize maturation, all broodstock were injected with LH-RH to induce spawning in April 2019, egg release was initiated in early May 2019. During that period, the animals were fed ad libitum with Vitalis Repro (Skretting, Stavanger, Norway), and egg production was monitored daily. When the total egg production stabilized, the egg batch was established at the beginning of May 2019. Eggs from the broodstock were collected and pooled for four consecutive days (4DL model) to maximize family representation. In total, 1.5–2 egg kg were incubated in cylinder conical tanks (650 L). Water conditions were as follows: temperature 20.0 °C, salinity 36%, and oxygen saturation was 100–120%. Thus, 633 offspring were individually tagged in the abdominal cavity for individual identification at 220 days post-hatching (dph), with a Passive Integrated Transporter (PIT, Trovan Daimler-Benz, United Kingdom), following the tagging protocol described by Navarro et al. (2006), and a sample of caudal fin was collected and preserved in absolute ethanol at room temperature for future DNA extraction. Thirty days later, fish were moved to different facilities of the company Avramar S.L. where they were reared in two different housing systems (HS): (1) 255 fish were allocated in a cage in the Mediterranean Sea (Villajoyosa, Alicante, Spain) under intensive conditions. The cage of 12 meters in diameter was anchored at a depth of 38 meters with a stock density of 10.6 kg/m^3 and water temperature of 20.4 °C (13–28 °C), salinity 34%, and oxygen saturation was 90%; (2) 378 fish were in a continental tank under intensive conditions at the Avramar S.L. facilities (Cabo Cope Aguilas, Murcia, Spain) with a stock density of 15 kg/m^3. The rectangular tank had a capacity of 20 m^3, with water temperature of 21.0 °C (19–23 °C), salinity 36%, and oxygen saturation was 100%.

At the end of the trial, a total of 633 fish reached harvest size (549 dph) and fish were slaughtered by immersion in ice cold water (hypothermia) and stored at 4 °C for 24 h for further processing.

4.2.3. Microsatellite Genotyping and Parental Assignment

DNA was extracted from the caudal fin using the DNA kit (E.Z.N.A.® Tissue, Norcross, U.S.A), and then kept at 4°C. Subsequently, DNA quantity and quality were determined with a NanoDrop™ 2000 spectrophotometer v.3.7 (Thermo Fisher Scientific, Wilmington, U.S.A.). All the fish, broodstock and offspring, were genotyped for 10 microsatellite loci multiplex (Table 1), using the Type-it Microsatellites PCR kit (QIAGEN®, Hilden, Germany).

The PCR was performed in a 12.5 µl reaction mix with a concentration of 10 µmol/l for each primer and 10 ng/ µl template DNA. The thermal profile included a pre-denaturation step of 95°C for 15 min followed by 32 cycles of denaturation-annealing-extension at 94°C for 30 s, 57°C for 90 s and 72°C for 1 min and one final elongation step at 60°C for 30 min. Amplicons were resolved by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystem, Foster City, CA, U.S.A.; (https://www.thermofisher.com/order/catalog/product/4405673?SID=srch-hj-4405673#/4405673?SID=srch-hj-4405673) using LIZ500 size standard marker. The fragment size was analyzed using Microsatellite analysis cloud (Thermo Fisher Scientific, Waltham, MA, USA, https://apps.thermofisher.com/editor-web/#/app/app-microsatellites-web), which was used for genotyping. For the parental assignment the exclusion method as implemented in VITASSING (v.8_2.1) software (Vandeputte et al., 2006) was used, where the assignment of parents, with an assignment error in the batch gate of 3.9% and batch tank of 1.8%. Finally, 616 fish were used in the different analyses, 245 from the cage and 371 from the tank.

Table 1. Microsatellite panel of meagre.

Meagre STR loci	M	F	Forward sequence (5´-3´)	Reverse sequence (5´-3´)	Reference
gCT15	$(GCT)^{7}$	5*NED	ATCCGGGCGTTACTACAGTC	GTTTCTCCACACAGTGCTTTTCAGA	Porta et al., 2010
UBA50	$(GT)^{26}$	5*NED	GCACAACTGCATCCCTTAGAT	GTTTAGAAGTGAAGACTGCGGACTG	Archangi et al., 2009
CA3	$(CA)^{12}$	5*NED	AAGTGGAGGCTCTTACATGAAAAC	GTGACAAATTGCCTTCTGTTTCTAC	Porta et al., 2010
GA17	$(GT)^{12}$	5*6-FAM	CTAGAGAAATTCATCCAGGGAAGTG	GTTTAGAGCAGAGAGTTAGCGGTTGTT	Porta et al., 2010
Cac mic 14	$(CT)_{12}$	5*6-FAM	ATCTTCTCCCCTCCGTCACT	CTGTGTTGTTAAGGCGCATC	Farias et al., 2006
GA2b	$(CA)^{26}$	5*PET	AAGTGTGGCGTCATTTCCTCT	GTATTGATGGATAGCAAGTGTCAGA	Porta et al., 2010
SOC405	$(CA)^{12}$	5*PET	AGCCTTTTGTTTAGTTTCCCTCAT	GGGGTGTAGCAGAACCACAC	Kathleen et al., 2003
SOC431	$(GT)^{26}$	5*VIC	GTGGTAGATGAAAACGTATAAAAGGAG	GTTTCATATATATAGTGTACAGCTCCAGCTTC	Kathleen et al., 2003
UBA53	$(CA)^{14}$	5*VIC	TACTTCCTTCTACCCCTAAGTCTGG	GACTTTCCAGTGTAGCTGTCGTTT	Archangi et al., 2009
SOC11	$(GA)_{11}$	5*VIC	GCCGAGTCACGAAGGAACAGAGAA	TGTCGTCTCATCTATCTCCATCTC	Saillant et al., 2004

M: motif of repetition; F: fluorescent tag.

4.2.4. Measurements

- Manual Growth (MG) Measurements

Body weight (BW) was measured with scales accurate to 1 g, and total length (TL) was measured with an ichthyometer with 1 mm divisions.

- Automatic Morphology (AM) Measurements

Immediately afterwards, each fish was photographed with a digital camera, side view, in a dark box with controlled light as per Navarro et al. (2016). Later, all pictures were analyzed, in a standardized way, by applying the automatic image analysis IMAFISH_ML software, developed in MatLab v.7.5 (The Math-Works Inc., Massachusetts, USA) (Navarro et al., 2016). All defined traits are shown in Table 2, and a fish picture indicating length and height measurement is depicted in Figure 1.

Table 2. The measured trait by IMAFISH_ML software.

Traits	Abbreviation	Description
Standard length (cm)	SL	Distance for X_1-X_4, within the horizontal axis (Figure 21).
Caudal peduncle height (cm)	CPH	Axis Y_3 (Figure 21).
Equidistant fish height C (cm)	FHC	TLL is divided into six equal parts, then heights of each one of these five points are measured FHA, FHB, FHC, FHD, and FHE are the axes a, b, c, d, and e, respectively (Figure 21).

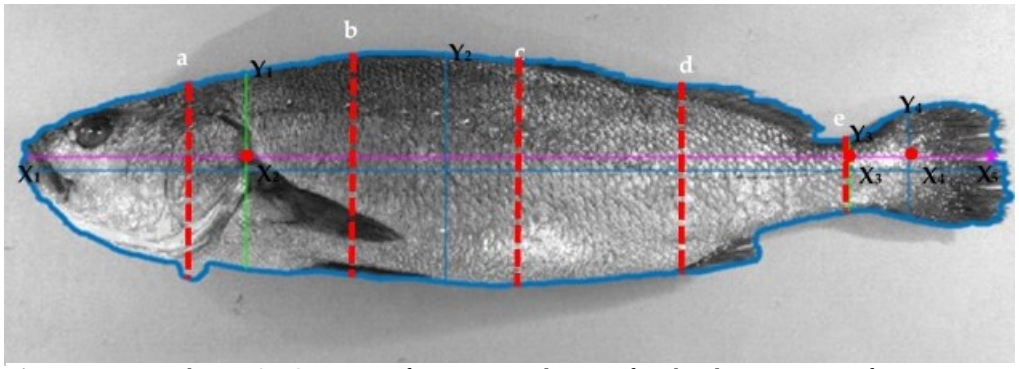

Figure 1. Image by IMAFISH_ML software. Lateral view, for the determination of Non-invasive Technological Traits (NiT) of *Argyrosomus regius*: points x_1, x_2, x_3, x_4, x_5 of the horizontal axis will be used to determine longitudinal traits; y_1, y_2, y_3, y_4 and a, b, c, d, e are dorsoventral axes, which will be used to determine height traits.

- Flesh Chemical Composition

The fish were then manually eviscerated, skinned, and filleted, with the fillets being frozen at −20 °C for further analysis of the flesh chemical composition.

For flesh chemical composition, the fillets were homogenized through a mixer, and the percentages of protein, fat, moisture, and collagen were estimated by the indirect method of near-infrared spectroscopy (near infrared spectroscopy, NIR), using FOODSCAN LAB equipment (FOSS IBERIA, Barcelona, Spain).

4.2.5 Statistical Data Analyses

- For phenotypical analysis

Numerical data for each trait were tested for normality and homogeneity of variances using SPSS® (v.26.0) (IBM Corp, 2017) and were analyzed with two General Linear Models (GLMs):

$$\begin{aligned} Y_{ij} &= \mu + HS_i + e_{ij}; \text{ for MG and AM measurements} \\ Y_{ij} &= \mu + HS_i + b*BW_j + e_{ij}; \text{ for flesh composition} \end{aligned} \quad (1)$$

in which Yij is an observation of an individual j in the housing system (HS) i, μ is the overall mean, HS is the effect of the environmental conditions because of the HS (i= cage or tank), b is the regression coefficient between the analyzed variable and the covariate BW, and eij is a random residual error.

- For genetic parameter estimates

For each combination of two traits, genetic parameters were estimated under a Bayesian approach using a two-trait animal mixed model which can be written as:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{Wp} + \boldsymbol{e}, \quad (2)$$

Where, $\boldsymbol{y}$ is the vector of data; $\boldsymbol{\beta}$ is the vector of systematic effects including the HS (2 levels: cage or tank) for all traits and the covariate body weight only for flesh composition traits; $\boldsymbol{u}$ is the vector of additive genetic effects; $\boldsymbol{p}$ is the permanent environmental effect of family-

HS; $\boldsymbol{e}$ is the residual; $\boldsymbol{X}$, $\boldsymbol{Z}$ and $\boldsymbol{W}$ are incidence matrices relating data with systematic effects and random additive genetic and permanent effects, respectively.

The systematic effects, $\boldsymbol{\beta}$, were assumed a priori to follow uniform distributions. The a priori distribution of the additive genetic effect was $p(\boldsymbol{a}|\boldsymbol{G}) \sim N(\boldsymbol{0}, \boldsymbol{G} \otimes \boldsymbol{A})$, where $\boldsymbol{G}$ is the 2 × 2 additive genetic covariance matrix between traits, $\boldsymbol{A}$ is the numerator relationship matrix, of dimension N, equal to the number of individuals in the pedigree and $\otimes$ is the Kronecker product. The a priori distribution of permanent environmental effects was, $p(\boldsymbol{p}|\boldsymbol{P}) \sim N\left(\boldsymbol{0}, \boldsymbol{P} \otimes \boldsymbol{I_p}\right)$, where $\boldsymbol{P}$ is the 2 x 2 covariance matrix of permanent environmental effects between traits and $\boldsymbol{I_p}$ is the identity matrix. Similarly, the distribution of the residual effects was $p(\boldsymbol{e}|\boldsymbol{R}) \sim N(\boldsymbol{0}, \boldsymbol{R} \otimes \boldsymbol{I_e})$, where $\boldsymbol{R}$ is the corresponding 2 x 2 residual covariance matrix between traits and $\boldsymbol{I_e}$ is the identity matrix. Bounded uniform priors were assumed for the elements of $\boldsymbol{G}$, $\boldsymbol{P}$ and $\boldsymbol{R}$.

$$\mathrm{G} = \begin{bmatrix} \sigma_{u1} & \sigma_{u1,u2} \\ \sigma_{u2,u1} & \sigma_{u2} \end{bmatrix}, P = \begin{bmatrix} \sigma_{p1} & \sigma_{p1,p2} \\ \sigma_{p2,p1} & \sigma_{p2} \end{bmatrix}, R = \begin{bmatrix} \sigma_{e1} & \sigma_{e1,e2} \\ \sigma_{e2,e1} & \sigma_{e2} \end{bmatrix} \qquad (3)$$

The marginal posterior distributions of all the unknowns were approximated by Gibbs sampling using the gibbs3f90 software (Misztal et al., 2015). Sampling processes of 200,000 iterations each were run. The first 50,000 iterations were discarded as burn-in and samples of the parameters of interest were saved every five iterations. The sampling variance of the chains was obtained by computing Monte Carlo standard errors. Statistics for the marginal posterior distributions were calculated directly from the samples using the R package "BOA" (R Development Core Team, 2016). The magnitude of estimated heritability was established following the classification recommended by Cardellino, R., Rovira (1987), as low (0.05–0.15), medium (0.20–0.40), high (0.45–0.60) and very high (>0.65). The magnitude of correlation was established following the classification of Navarro et al. (2009a), low (0–0.40), medium (0.45–0.55) and high (0.60–1), regardless of whether they were positive or negative.

4.3. Results

4.3.1. Phenotyping

The phenotyping results for MG (BW and TL) and AM measurements (SL, CPH, and FHC) are shown in Table 2. The fish reared in the cage showed higher BW (38% heavier than the average), TL, SL, CPH, and FHC (15, 16, 15, and 18% greater than the average, respectively), than those in the tank. The much higher increase in BW than length or height indicated that the growth was more in volume than in the area for this weight range.

To know if the meagre's shape is affected by BW, the SL/FHC ratio was calculated including BW as covariate (Table 2). Therefore, we observed that when BW was adjusted to average BW equal to 995 g, there was no difference between fish reared in the cage or in the tank. However, BW had a significant negative effect on the SL/FHC ratio although it was very reduced, thus when fish increased their weight by 100 g this ratio decreased 1.61×10^{-2} (footnote Table 2); increased BW in meagre involved slight changes in shape, gaining more in height than in length. For the harvest BW, the phenotypical variation for this ratio ranged from a minimum and a maximum value of 3.11 to 4.79, with 95% of the fish being in the interval [3.93–4.05], so the meagre were four times longer than they were wide.

Table 2. Manual growth (body weight and total length) and automatic morphology measurements (by IMAFISH_ML, software) (least square means, LSM, ± standard error, SE), for meagre at 549 days post hatching raised in two different environments

Environment	Cage			Tank		
	n	LSM	S.E.	n	LSM	S.E.
BW (g)	245	1233[a]	18.3	371	839[b]	14.8
TL (cm)	245	42.7[a]	0.27	371	36.8[b]	0.22
SL (cm)	245	40.7[a]	0.35	371	34.7[b]	0.28
CPH (cm)	245	3.51[a]	0.03	371	3.01[b]	0.02
FHC (cm)	245	10.3[a]	0.08	371	8.59[b]	0.07
SL/FHC*	245	3.96	0.02	371	4.03	0.01

ab: different superscripts within each row indicate significant differences between housing system ($P < 0.05$); BW (Body weight), TL (Total Length), SL (Standard length), CPH (Caudal peduncle height) and FHC (Equidistant fish height C). It was adjusted to BW (mean weight of 995 g) regression coefficient = $-1.61*10^{-4} \pm 3.04*10^{-5}$.

The flesh composition (moisture, protein, fat, and collagen percentages) is shown in Table 3. The BW showed a negative effect on moisture, and positive on the fat percentage; thus when the fish weight increased 100 g, the moisture decreased 0.3% and the fat increased 0.2%. When flesh composition was adjusted to an average BW of 995 g, the cage fish showed a higher

fat percentage (33% better than the average) and lower protein percentage (−8.7% better than the average) than the tank fish.

Table 3. Flesh composition (protein, moisture, fat, and collagen percentages) (least square means, LSM, ± standard error, S.E.), for meagre at 549 days post hatching raised in two different environments.

Environment	Cage			Tank			Covariate BW	
	n	LSM	S.E.	n	LSM	S.E.	b	S.E.
Moisture (%)	245	74.0	0.10	371	73.9	0.08	-0.003*	0.00
Protein (%)	245	18.5[a]	0.09	371	20.2[b]	0.07	-0.000	0.00
Fat (%)	245	5.62[a]	0.12	371	4.02[b]	0.09	0.002*	0.00
Collagen (%)	245	1.00	0.03	371	1.00	0.02	<0.000	<0.000

ab: different superscripts within each row indicate significant differences between housing system ($P < 0.05$); b = regression coefficient for moisture, protein, lipids content, and collagen were adjusted to average BW 995 g; * = covariate was significant ($P < 0.05$).

4.3.2. Microsatellite Genotyping and parental assignment

For the cage fish, 97.2% of the offspring were assigned, of which 94.5% were assigned to a single couple of parents. In the case of the tank fish, a 98.9% parental assignment was obtained, of which 97.9% were assigned to a single couple. After the assignment, unequal breeder contribution was observed. In the cage fish, one out of five females produced 45.3% of the offspring although all the females contributed to the offspring, and one out of four males contributed with 59.5% of the offspring and all the males contributed. Similarly, in the tank fish, two out of five females contributed with 55.5% of the offspring and all females contributed, whilst two out of four males contributed with 63.8% of the offspring and all the males contributed. Pedigree construction using selected highly informative microsatellite markers yielded 20 full-sib families for the cage fish with a mean of 15.06 sibs (range 2-58 sibs), and in the tank fish, it produced 20 full-sib with a mean of 19.47 sibs (range 7-60 sibs).

4.3.3. Heritabilities and correlations

- Heritabilities

Heritabilities estimated for each trait and genetic and phenotypic correlations between traits are shown in Table 4. In general, heritability estimates showed medium and low values with a high standard error, so the results should be viewed with caution. In the case of MG traits, the heritabilities were medium for BW and TL. For AM traits, SL and FHC showed medium heritability and CPH and SL/FHC low. For flesh composition, fat and moisture percentages showed medium heritability and protein and collagen low heritability.

- Correlations

  - Within group of traits:

The phenotypic correlations were estimated with high accuracy. They were positive and high between MG traits, BW–TL, and between AM traits (SL, FHC, and CPH), except for the SL/FHC ratio which was almost null with length measurements (TL and SL) and low and negative with height measurements (CPH and FHC). For flesh composition they were positive and low for collagen–moisture, negative and high for fat–moisture, medium for fat–protein, and negative and low for protein–collagen, protein–moisture, and collagen–fat.

The genetic correlations between the MG traits, BW–TL, were very high. Between the AM traits they were positive and high for all the traits (SL, FHC, and CPH) except for SL/FHC, which showed similar patterns but were estimated with low accuracy.

In the case of flesh composition traits, most of them were estimated with little accuracy, with a high standard error, except for the correlation between fat and moisture, and therefore safely interpreting the corresponding genetic correlations is difficult. Taking this consideration into account, the genetic correlations estimated were positive and low for moisture–collagen, negative and high for fat–moisture, negative and medium for fat–protein and fat–collagen, and negative and low for protein–collagen and moisture–protein. Genetic correlations were in the same order as the phenotypic correlations.

  - Between groups of traits:

Since AM traits (SL, CPH, and FHC) are another way to analyze the MG (BW and TL), the phenotypic correlations between them were positive and high in all the cases, except for the SL/FHC ratio, which were low and negative. The BW correlations were higher with height than with length measurements. Considering these results, at this weight range, the fish is growing slightly more in height than in length.

Regarding MG traits and flesh composition, phenotypic correlations were positive and medium for fat, negative and medium for moisture and low for protein, and almost null for collagen. For the AM traits and flesh composition the phenotypic correlations followed a similar pattern to the MG traits and flesh composition, apart from the SL/FHC ratio. In the case of the SL/FHC ratio, the correlations were almost null with protein and collagen, medium with fat and moisture, and negative for fat and positive for moisture.

Genetic correlations between MG (BW and TL) and AM traits (SL, CPH, and FHC) were positive and very high for all the possible combinations, except for the SL/FHC ratio, which were negative with BW and null with TL but estimated with high standard errors.

Genetic correlations between MG traits (BW and TL) and flesh composition were estimated with low accuracy and thus interpreting them safely becomes difficult. Most of these correlations were very low except for protein, with both BW and TL showing a negative and medium value.

Genetic correlations between the AM traits and flesh composition showed higher values than those with the MG traits, highlighting the negative correlations between fat and SL, CPH, and SL/FHC and, conversely, the positive correlations between moisture and SL, CPH, and SL/FHC. Protein showed negative correlations with all the AM traits and collagen showed negative correlations with SL and FHC, and positive with CPH and SL/FHC, although all of them had high standard errors.

Table 4. Heritabilities (in bold at diagonal, with standard error), phenotypic correlations (below the diagonal in italics, with standard error) and genetic correlations (above the diagonal, with standard error) for manual growth, automatic morphology and flesh composition measurements, in meagre at harvest size (549 dph).

Traits	BW	TL	SL	CPH	FHC	SL/FHC	Moisture	Protein	Fat	Collagen
BW	**0.42 ± 0.24**	0.96 ± 0.06	0.89 ± 0.19	0.90 ± 0.16	0.89 ± 0.18	−0.13 ± 0.64	0.14 ± 0.53	−0.43 ± 0.53	−0.09 ± 0.50	0.05 ± 0.58
TL	*0.91 ± 0.01*	**0.38 ± 0.22**	0.90 ± 0.16	0.88 ± 0.18	0.86 ± 0.20	−0.00 ± 0.62	0.09 ± 0.50	−0.43 ± 0.49	−0.01 ± 0.45	−0.04 ± 0.52
SL	*0.68 ± 0.04*	*0.75 ± 0.04*	**0.32 ± 0.23**	0.90 ± 0.21	0.95 ± 0.11	0.07 ± 0.71	0.29 ± 0.60	−0.22 ± 0.66	−0.37 ± 0.57	−0.34 ± 0.60
CPH	*0.66 ± 0.04*	*0.65 ± 0.05*	*0.81 ± 0.03*	**0.19 ± 0.16**	0.79 ± 0.03	0.08 ± 0.69	0.31 ± 0.57	−0.20 ± 0.65	−0.40 ± 0.53	0.32 ± 0.60
FHC	*0.74 ± 0.03*	*0.74 ± 0.04*	*0.94 ± 0.01*	*0.83 ± 0.02*	**0.39 ± 0.20**	−0.33 ± 0.65	−0.03 ± 0.66	−0.10 ± 0.68	−0.08 ± 0.63	−0.44 ± 0.57
SL/FHC	*−0.30 ± 0.10*	*−0.08 ± 0.11*	*0.03 ± 0.11*	*−0.17 ± 0.10*	*−0.31 ± 0.10*	**0.16 ± 0.15**	0.76 ± 0.37	−0.36 ± 0.64	−0.72 ± 0.39	0.26 ± 0.65
Moisture	*−0.46 ± 0.10*	*−0.41 ± 0.10*	*−0.28 ± 0.10*	*−0.31 ± 0.09*	*−0.36 ± 0.10*	*0.25 ± 0.07*	**0.32 ± 0.21**	−0.27 ± 0.62	−0.95 ± 0.07	0.08 ± 0.61
Protein	*−0.02 ± 0.12*	*0.02 ± 0.12*	*0.06 ± 0.12*	*0.09 ± 0.12*	*0.03 ± 0.12*	*0.01 ± 0.11*	*−0.11 ± 0.11*	**0.15 ± 0.14**	−0.17 ± 0.60	−0.15 ± 0.65
Fat	*0.37 ± 0.10*	*0.25 ± 0.12*	*0.17 ± 0.11*	*0.12 ± 0.10*	*0.19 ± 0.12*	*−0.21 ± 0.09*	*−0.82 ± 0.03*	*−0.38 ± 0.09*	**0.30 ± 0.20**	−0.01 ± 0.59
Collagen	*−0.01 ± 0.12*	*−0.02 ± 0.12*	*−0.04 ± 0.12*	*−0.04 ± 0.11*	*−0.04 ± 0.12*	*0.03 ± 0.10*	*0.17 ± 0.08*	*−0.24 ± 0.08*	*−0.06 ± 0.10*	**0.15 ± 0.16**

BW = body weight, g; TL = total length, cm; SL = standard length, cm; CPH = caudal peduncle height, cm; FHC = equidistant fish height C, cm; flesh composition (moisture, protein, fat, and collagen, in %). Different colors represent correlations within the same group traits (gray), correlations between AM traits and MG and flesh composition traits (yellow), and between MG and flesh composition traits (green).

4.4. Discussion

This study is one of the first in which genetic parameters for growth, morphological traits, and flesh composition (fat, protein, moisture, and collagen percentages) have been studied on meagre (*Argyrosomus regius*) from two batches, one reared in a cage and the other in a tank. Growth and morphological traits play a very important role in terms of economic performance in the aquaculture industry. Flesh composition, especially fat content and its fatty acid profile, is one of the characteristics that consumers appreciate most.

The first step when estimating genetic parameters was the design of the paternity. Using microsatellite markers is a widespread method for parental assignment (García-Celdrán et al., 2015; Lee-Montero et al., 2013; Navarro et al., 2008; Nousias et al., 2020; Villanueva et al., 2002). In our case, with a 10-microsatellite panel we had a slightly higher parental assignment than Nousias et al. (2020) who obtained assignments of 87.5% and 95% for two batches. A crucial factor in obtaining a high percentage of assignment in a population is that the set of selected markers must be highly polymorphic. In our study, two of them (gCT15 and CA3) were not, and they could be removed in the microsatellite markers panel. The remaining eight microsatellites proved to be enough to obtain a successful assignment.

After the construction of the pedigree, measured traits were phenotypically analyzed. For growth, our results highlighted the high growth rate of meagre, as in Fountoulaki et al. (2017), thereby making it very interesting for the aquaculture industry. An important factor affecting growth rate is the stock density. Thus, we observed lower BW for fish in the tank than in the cage, which is probably mostly due to the higher stock density in the tank. Piccolo et al. (2008) observed a BW of about 830 g and a TL of 42 cm in meagre raised in cages with a similar stock density (9.8 kg/m^3) to that in our work for 15 months, and Poli et al. (2003) with fish reared in tanks with high stock density about 44 kg/m^3, found that the BW was 935.5 g, 1199.6 g, and 1502.5 g when they were 24, 26, and 30 months old, respectively. Breeders are usually kept in tanks, therefore the environmental conditions in the tank are important to enable the breeders to express their best performance.

Regarding the morphological traits measured in this study, this is the first attempt to describe the shape in meagre and relate it to growth traits. Elalfy et al. (2021) also used IMAFISH_ML software as a tool in breeding programs to obtain a detailed description about the fish morphology in gilthead seabream. Since image analysis is a non-invasive measurement of individuals, it can help us to select a fish as a future breeder by its own data and not through

its offspring, which is more expensive and involves a longer process. In addition, the image provides us with a larger amount of objective information than can be gained from manual measurements because it does not depend on a person.

For fillet composition of commercially sized meagre, the fillet fat percentage in previous studies was slightly lower than in our work, ranging between 0.6–1% (Fountoulaki et al., 2017; Giogios et al., 2013) and 3% (Piccolo et al., 2008) depending on the diet's fat content. Protein percentage changed very little and was usually 20–21% (Fountoulaki et al., 2017; Giogios et al., 2013; Piccolo et al., 2008) depending on the diet. In our work, the cage fish showed a higher fat percentage and a lower protein percentage (18.5%) in comparison with the tank fish and with other works. The higher fat percentage in the cage fish is likely due to a lower temperature that increased appetite and energy reserves. In addition, a pronounced seasonality has been observed in fillet fat content, which reached a maximum with the replenishment of body fat stores in early autumn (Ballester-Lozano et al., 2011) when our fish were slaughtered. Additionally, the methodology to measure fillet fat content in our work (NIR) differed from that used in other studies (Folch et al., 1957). Furthermore, the fillet fat percentage showed a positive phenotypic correlation with BW in accordance with Fountoulaki et al. (2017). In gilthead seabream, Elalfy et al. (2021) observed an effect of the HS on protein percentage, thus protein was 19.38% when fish were cage-reared and 20.84% when estuary-reared.

In the end, the genetic component in the variation of the measured traits was analyzed. For MG and AM traits, medium heritabilities were observed for all of them except for CPH and the SL/FHC ratio, which were low. In meagre, Nousias et al. (2020) observed very high heritability for BW (0.62) and TL (0.64) in populations without previous selection and under industrial farming conditions, as in our study. There are no further references about additive genetic variation in meagre. In other marine fish, Vallecillos et al. (2021) obtained medium heritabilities for body weight (0.20) in juvenile gilthead seabream; similar values were obtained for BW heritability in several studies of different species (Carballo et al., 2020; Freitas et al., 2021; García-Celdrán et al., 2015; Pattarapanyawong et al., 2021).

Genetic variation has not been studied previously for AM traits in meagre (SL, CPH, and FHC). Elalfy et al. (2021) obtained a medium heritability for the same variables but in gilthead seabream. In our case, we obtained a medium heritability for SL and FHC variables but the heritability for CPH was low. Moreover, the SL, CPH, and FHC measurements were closely related to the MG traits phenotypically and genetically, highlighting that they could replace MG measurements as a selection criterion in a breeding program.

Flesh composition traits, together with other quality parameters of the fish, are becoming more relevant in genetic breeding programs (Janssen et al., 2017). This is reinforced by the consumer, since every day they are more concerned about the quality of the fish farming products usually being comparable to wild ones. In this sense, it is important to know about the genetic variation of these traits. In our study, fillet fat and moisture percentages showed medium heritability, and protein and collagen percentages were low. To the best of our knowledge there has been no work about fillet composition heritabilities in meagre, although similar results have been observed in other species (Elalfy et al., 2021; García-Celdrán et al., 2015; Noble et al., 2020; Sang et al., 2020).

Genetic correlations for MG traits (BW and TL) were high and positive in accordance with Nousias et al. (2020). There has been no work about AM traits in meagre, but Elalfy et al. (2021) studied them in gilthead seabream and observed high and positive correlations between MG and AM traits, as happened in our work. Therefore, in a breeding program improving one of them, the rest of the traits will improve indirectly. However, the phenotypic and genetic correlation was negative for the SL/FHC ratio, thus this ratio could be studied as it changes with the selection process.

Genetic correlations between the MG or AM traits and flesh composition were not estimated accurately, due to the limited amount of data. The most interesting genetic correlations are between the fillet fat percentage and the MG and AM traits. In gilthead seabream, Elalfy et al. (2021) and Lee_Montero et al. (2012) observed a positive and medium correlation between growth and fillet fat percentage, suggesting that selection to improve growth traits could lead to fish with a higher fillet fat percentage. Further studies are needed to improve estimates for genetic correlations and to continue with the analysis of other carcass and meat quality traits.

4.5. Conclusions

Attention should be paid to the housing conditions for raising the fish, as the fish in the tank showed worse results for growth, and breeders are typically kept in tanks. Medium heritabilities for growth, morphological traits, and fillet fat percentage revealed them as a possible criterion to be included in a breeding program. Image analysis to describe fish morphology could replace growth measurements in a breeding program, since the amount of information obtained is very high and objective, changes in fish morphology could be observed,

and these were positive and highly correlated with growth traits. An increase in the fillet fat percentage could be expected with the selection process for fish growth due to the medium and positive phenotypic correlation between the fillet fat percentage and growth and morphology traits, although genetic correlations with flesh composition were not estimated accurately. Further studies are necessary to delve deeper into these aspects and into other quality traits.

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Chapter 4. Final conclusions

The general conclusions are as follows:

I. The approach of using immunological markers under innate conditions, such as peroxidase activity, in breeding programs allows us to select individuals by their own data without having to evaluate them through their offspring. Using these markers, together with other criteria, such as weight or the absence of malformations, will result in animals that are more resistant to various diseases and adverse environmental conditions.

II. Selection for growth can lead to an increase in fat content and a reduction in the percentage of polyunsaturated fatty acids. The average heritability shown by some fatty acids, as well as the n3/n6 ratio, should be taken into account. Consideration of quality traits, such as fillet fat content and fatty acid profile, should be part of genetic improvement programs.

III. A panel of microsatellite markers has been designed in corvina that will allow us to assign parentage. We have started with the study of genetic parameters of growth and chemical composition of the fillet, the results of which indicate that genetic progress can be expected for these characteristics. Image analysis has proven to be a very useful tool in genetic improvement programs for fish growth and morphology.

With this doctoral thesis we hope to have provided information that will help in the approach to the new challenges of aquaculture and the competitiveness of the sector. However, we are aware that future work is necessary in order to continue advancing in this field.

Chapter 5. Appendix

Table S1: Allele frequency for different microsatellites of the offspring population, in 616 fish.

UBA50		gA17		Cacmic 14		gA2B		SOC 405		UBA53		SOC 431		Soc 011	
Alleles	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**	**Alleles**	**Freq.**
128	0,0073	80	0,1761	132	0,0317	86	0,0049	112	0,0998	86	0,0008	124	0,0016	250	0,0008
130	0,0170	82	0,4683	134	0,4383	88	0,2833	114	0,8255	88	0,0008	132	0,0779	252	0,2716
132	0,0982	84	0,1526	136	0,0024	90	0,6274	118	0,0065	90	0,0008	134	0,6721	254	0,1788
134	0,3466	86	0,0016	138	0,0008	92	0,0024	120	0,0438	102	0,0008	136	0,2086	256	0,1349
136	0,1558	90	0,0057	140	0,2005	94	0,0154	124	0,0244	106	0,0041	140	0,0097	258	0,0260
138	0,0146	92	0,1940	142	0,0593	98	0,0008			108	0,0529	142	0,0073	260	0,0008
140	0,0519			144	0,0747	104	0,0536			110	0,3953	144	0,0105	262	0,1732
142	0,0528			146	0,0170	106	0,0073			112	0,0723	148	0,0122	264	0,0057
144	0,0373			148	0,1753	110	0,0049			114	0,4722			266	0,2081
146	0,0349														
148	0,0057														
150	0,0138														
152	0,1640														

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2020 JOURNAL IMPACT FACTOR

4.242

View calculation

JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS

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View calculation

Journal Impact Factor Trend 2020

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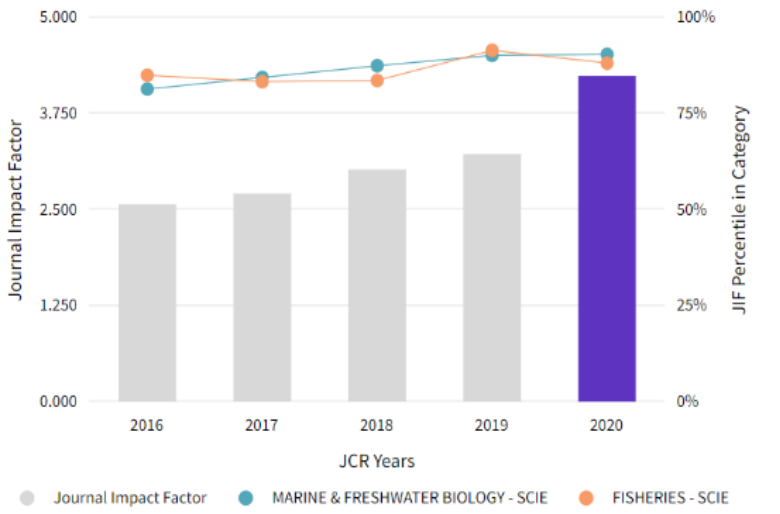

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EDITION
Science Citation Index Expanded (SCIE)

CATEGORY
FISHERIES
7/55

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2020	7/55	Q1	88.18
2019	5/53	Q1	91.51
2018	9/52	Q1	83.65
2017	9/51	Q1	83.33
2016	8/50	Q1	85.00

EDITION
Science Citation Index Expanded (SCIE)

CATEGORY
MARINE & FRESHWATER BIOLOGY
11/110

JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
2020	11/110	Q1	90.45
2019	11/107	Q1	90.19
2018	14/108	Q1	87.50
2017	17/106	Q1	84.43
2016	20/105	Q1	81.43

Paper 2.-: Antonio Vallecillos, María Marín, Martina Bortoletti, Javier López, Juan M. Afonso, Guillermo Ramis, Marta Arizcun, Emilio María-Dolores y Eva Armero, 2021. Genetic Analysis of the Fatty Acid Profile in Gilthead Seabream (*Sparus aurata*). Animals 11, 2889. https://doi.org/10.3390/ani11102889

Paper 4.-: Antonio Vallecillos, Emilio María-Dolores, Javier Villa, Francisco Miguel Rueda, José Carrillo, Guillermo Ramis, Mohamed Soula, Juan Manuel Afonso y Eva Armero, 2021. Phenotypic and Genetic Components for Growth, Morphology and Flesh-Quality Traits of Meagre (*Argyrosomus regius*) Reared in Tank and Sea Cage. Animals 11, 3285. https://doi.org/10.3390/ani11113285

Animals Statistics

Overview

Animals in Numbers

Animals (ISSN: 2076-2615)

Founded: 2011 (Volumes: 12)

9,199 articles published so far

1080 articles have been cited 10 times or more

H5-Index: 38 (Veterinary Medicine)

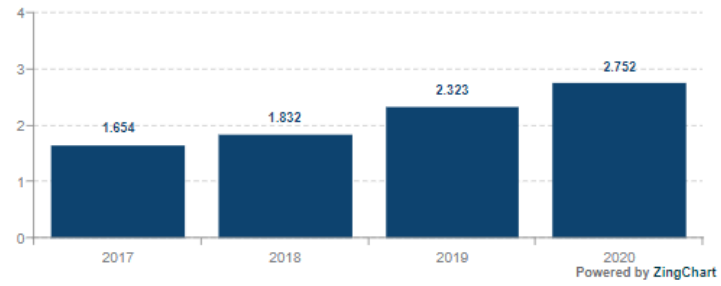
Impact Factor

Current Impact Factor: 2.752

5-year Impact Factor: 2.942

JCR category rank: Q1: Veterinary Sciences | Q1: Agriculture, Dairy & Animal Science

Impact Factor



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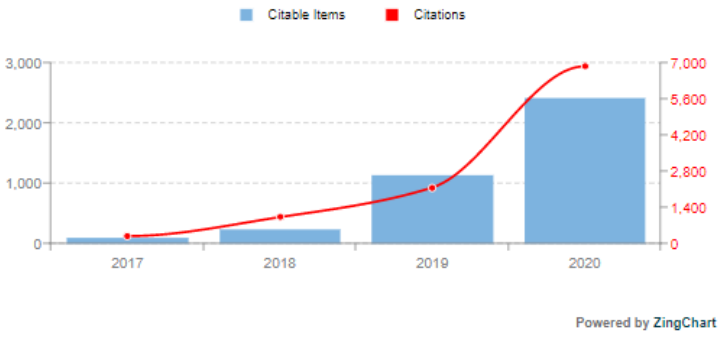

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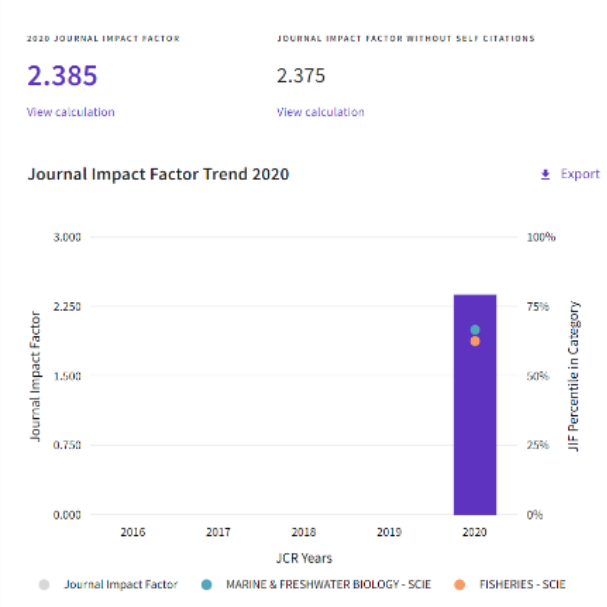

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EDITION	CATEGORY	JCR YEAR	JIF RANK	JIF QUARTILE	JIF PERCENTILE
Science Citation Index Expanded (SCIE)	FISHERIES 21/55	2020	21/55	Q2	62.73
Science Citation Index Expanded (SCIE)	MARINE & FRESHWATER BIOLOGY 37/110	2020	37/110	Q2	66.82

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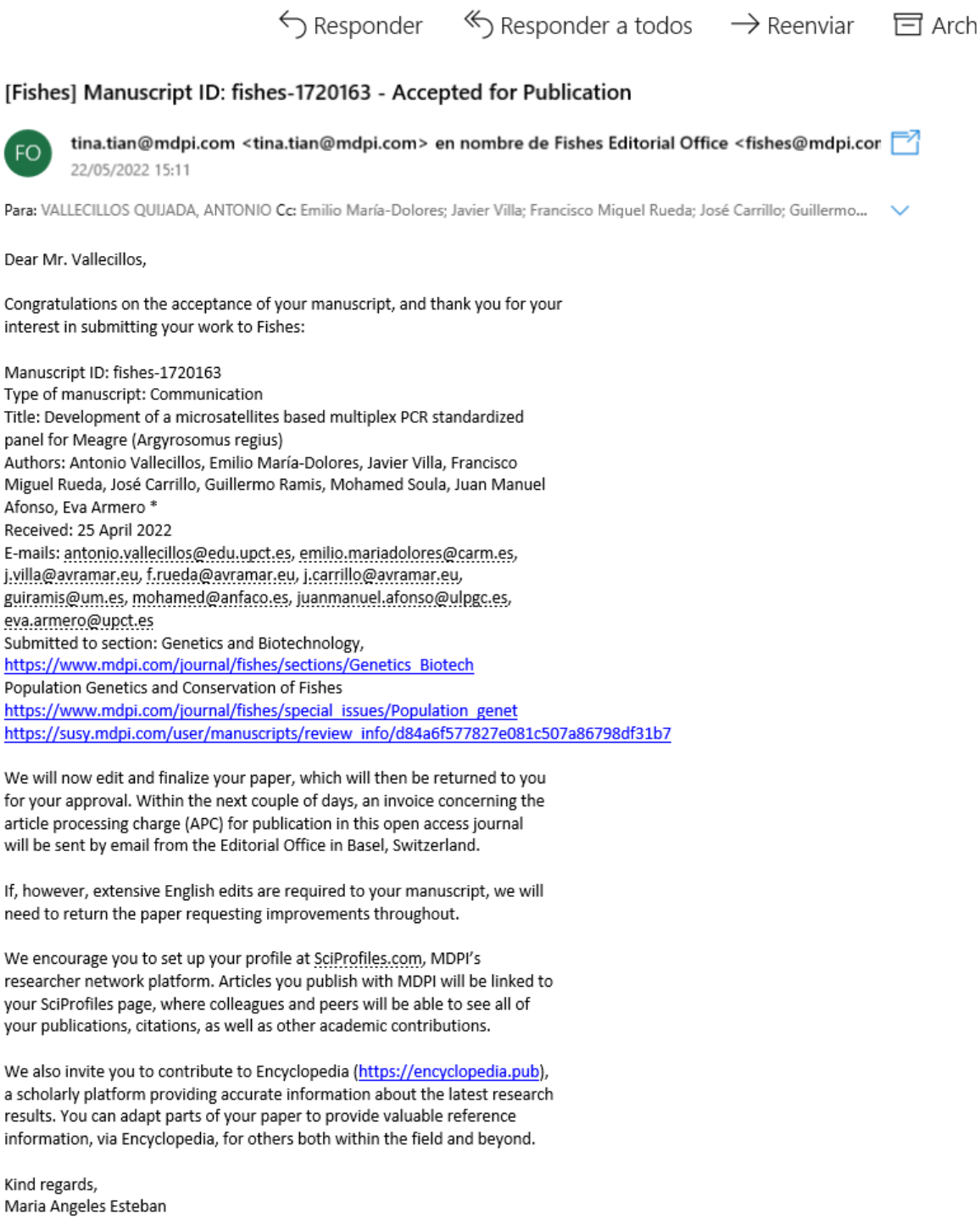

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