Universidad Politécnica de Cartagena

UPCT

Escuela Técnicas Superior de Ingeniería Agronómica

ETSIa

Máster Universitario en Ingeniería Agronómica

Parámetros genéticos para la resistencia a *Photobacterium damselae*, marcadores inmunológicos y peso corporal en Dorada (*Sparus aurata* L.)

Autor: Antonio Vallecillos Quijada

Directora: Eva Armero Ibáñez

Codirector: Emilio María-Dolores Pedrero

Cartagena, diciembre de 2020

Índice contenido

Índice figuras

Índice tablas

RESUMEN

En dos poblaciones de juveniles de dorada (*Sparus aurata* L.): F2_ATL y F0_MED, a los 272 días post-eclosión, se llevó a cabo un reto de infección a *Photobacterium damselae* subsp. *piscicida* (*Phb*)Previa a la infección, los individuos fueron pesados y se obtuvo una muestra de plasma de cada individuo para medir marcadores inmunológicos (actividad peroxidasa, actividad bacteriana, e inmunoglobulinas IgM), seguidamente los peces fueron inoculados con la bacteria *Phb*. A partir de este momento, se fueron registrando los peces que morían durante 9 días y los días hasta la muerte de cada uno de los individuos. Las heredabilidades estimadas para el peso corporal y la resistencia fueron moderadas, sin embargo, para los días hasta la muerte fue baja. Respecto a las heredabilidades de los marcadores inmunológicos, la estimación para actividad peroxidasa fue moderada y para los niveles de IgM y de actividad bactericida fue baja. La correlación genética de peso corporal con resistencia a *Phd* y días hasta la muerte fue alta y positiva, mientras que con actividad peroxidasa y los niveles de IgM tendía a ser positiva, pero estas estimaciones no fueron precisas. Por el contrario, la correlación genética entre peso corporal y actividad bactericida fue alta pero negativa. Respecto a correlación genética entre resistencia a *Phd* y marcadores inmunes fue alta, positiva con actividad peroxidasa y negativa con niveles de IgM y actividad bactericida. A partir de estos resultados, algunos marcadores inmunes, junto con el peso del pez y la ausencia de deformaciones, podrían ser incluidos en un programa de mejora genética para conseguir peces que sean capaces de hacer frente a enfermedades y desafíos ambientales.

ABSTRACT

A challenge test for *Photobacterium damselae* subsp. *piscicida* (*Phd*) resistance in two juvenile populations of gilthead sea bream (*Sparus aurata* L.): F2_ATL and F0_MED was carried out. At 272 days post-hatching, fish were sampled for weight and a plasma sample was collected to measure *humoral immune markers* (*peroxidase activity*, *bactericidal activity* and *IgM immunoglobulins levels*), and after fish were inoculated with bacteria *Phd*. From this time, survival fish were recorded for 9 days, and days to death was registered. Heritabilities for *body weight* and for *Phd resistance* were moderate, however for *days to death* was low. Regarding *humoral immune markers*, for *peroxidase activity* was moderate, and for *IgM levels* and for *bactericidal activity* was low. Genetic correlations for *body weight* with *Phd resistance* and *days to death* were high and positive, while with *peroxidase activity* and *IgM levels* tended to be positive but these estimates were not accurate. On the contrary, genetic correlation between *body weight* and *bactericidal activity* was high but negative. Regarding genetic correlations between *Phd resistance* and *humoral immune markers* were very high, positive with *peroxidase activity* and negative with *IgM levels* and *bactericidal activity*. Some *humoral immune markers*, along with the weight of the fish and the absence of deformities, could be included in a selective breeding program to raise fish that are capable of coping with diseases and environmental challenges.

ESTRUCTURA DEL TFM

El presente TFM se centra en el desarrollo de un artículo científico que ha sido enviado a la revista "Animals", edición especial "Aquatic Animals: new horizons for blue growth" (Índice de impacto:2.323, Q1). Si bien, se ha desarrollado la introducción en español con mayor profundidad y se han realizado unas conclusiones en español más detalladas.

INTRODUCCIÓN

La dorada (*Sparus aurata*) es una de las especies más importantes en el área Mediterránea tanto para la pesca como para la acuicultura, con una producción acuícola total de 252.406 toneladas en el año 2019. La producción de la dorada en acuicultura la lideran 20 países, entro los que cabe destacar los de mayor producción; con 85.000 t Turquía, seguido de Grecia con 65.300 t, Egipto con 36.000 t, Túnez con 16.000 t y En quinto lugar España con un total de 13.521 t (Figura 1). Con una producción de juveniles en Europa estimada de 701.511 millones de unidades (incluyendo a Turquía) [1].

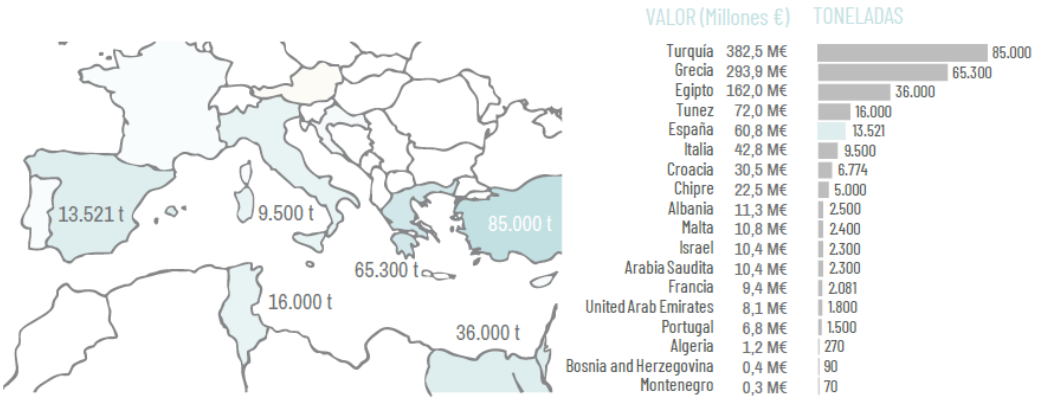


FIGURE 1. PRODUCCIÓN DE DORADA DE ACUICULTURA EN EL ÁREA MEDITERRÁNEA EN 2019. SOBRE DATOS DE FAO, FEAP Y APROMAR.

En cuanto a datos de producción a nivel nacional, se estima que fue de 13.521 t en 2019, con un descenso de un 9,4% con respecto a 2018. Cabe destacar que para 2020 se prevé un descenso en la producción de dorada de un 27,3%, todo ello a causa de los diversos fenómenos meteorológicos que están azotando las costas españolas, como sucedió con la tormenta "Gloria" y pérdidas causadas por patologías en el último trimestre de 2019 y el primero de 2020. Entre las comunidades con mayor producción de dorada tenemos a la Comunidad Valenciana con 6.629 t, seguido por Murcia con 2.906 t, Canarias con 2.380

t y en cuarta posición Andalucía con 1.606 t (Figura 2). Con respecto a la producción de juveniles, hablamos de 30 millones de unidades [1].

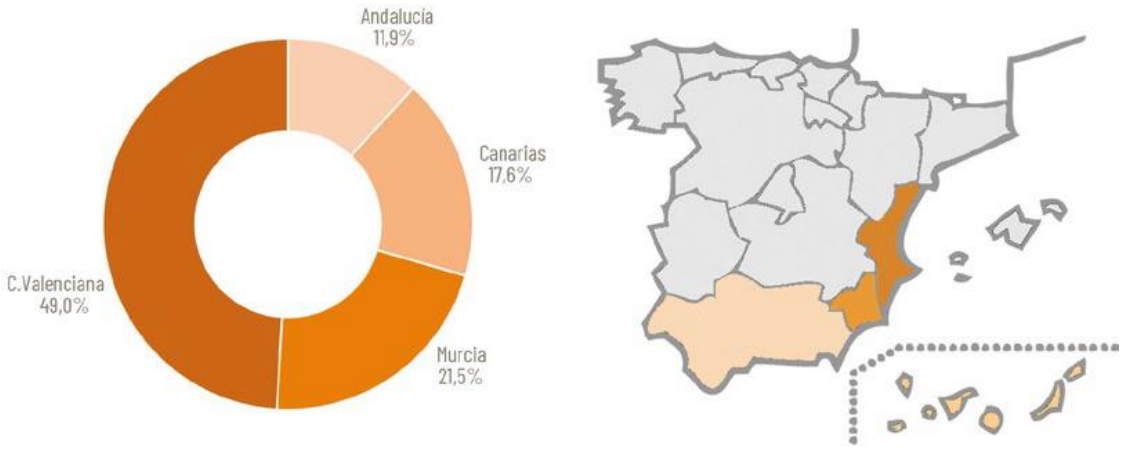


FIGURE 2. PRODUCCIÓN (TONELADAS) DE DORADA POR COMUNIDAD AUTÓNOMA EN 2019.

Como ya hemos comentado anteriormente, los patógenos son algunos de los causantes de las grandes pérdidas económicas que sufre el sector de la acuicultura, entre los que cabe destacar *Vibrio* (67.8%), *Pseudomonas* (13.5%), *Photobacterium damselae* subsp. *piscicida* (6.7%), *Cytophaga/Flexibacter-like bacteria* (4.8%), *Aeromonas* (0.5%) y *Gram-positive bacteria* (6.7%) que afectan a la producción de dorada en los porcentajes indicados. Teniendo en cuanta que los mayores porcentajes corresponden a *Vibrio* y *Photobacterium damselae* subsp. *Piscicida (Phd),* dando lugar a un alto porcentaje de mortalidad resultando en unas graves pérdidas para la industria acuícola [2].

A continuación, nos vamos a centrar en el patógeno objeto de nuestro estudio *Phd. Phd,* se localizó por primera vez en poblaciones silvestres de perca blanca (*Perca fluviatilis*) y lubina rayada (*Morone saxatilis*), teniendo en cuenta que actualmente una gran variedad de peces marinos son huésped de este patógeno. *Phd* es la causante de la "pseudotuberculosis" que se reconoce como lesiones de tipo granulomatoso en el bazo y el riñón de los peces afectados. Los síntomas externos suelen pasar desapercibidos y por lo general no se observan lesiones externas, algunas de las causas a valorar son pérdida de peso, oscurecimiento de la piel, necrosis localizada en las branquias y distensión del estómago, e internamente son tumores de entre 0.3-0.5 mm en algunos órganos y una

congestión y dilatación del tracto digestivo (Figura 3), todo ello depende de si la enfermedad es aguda o crónica [3]. *Phd* parece ser más frecuente durante los meses con elevadas temperaturas y salinidad de un 20-30 ‰, comúnmente suele producirse durante los meses de verano.

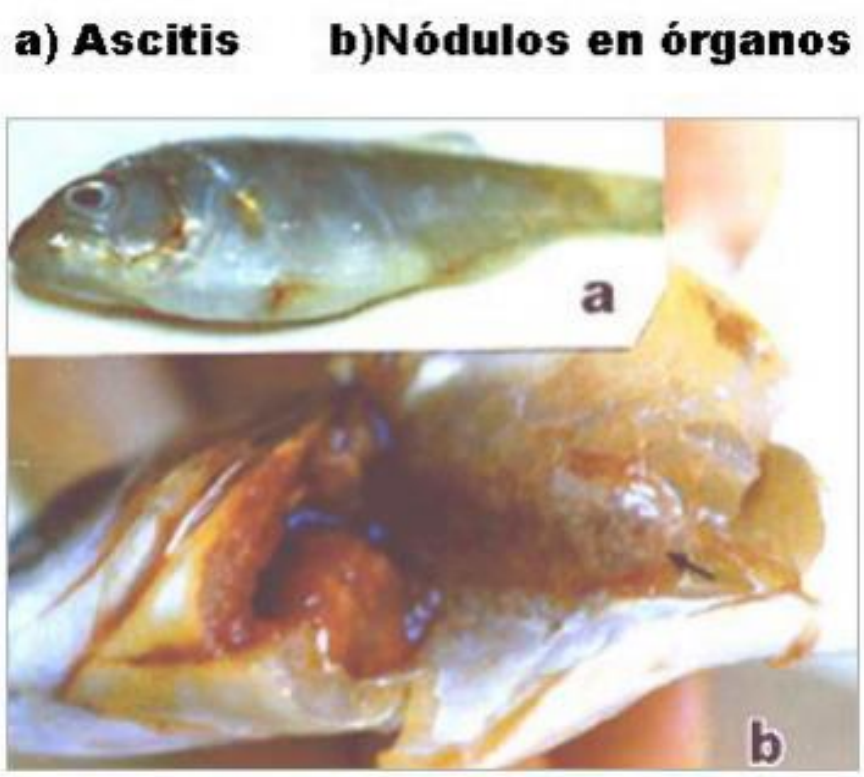


FIGURE 3. EFECTOS DE EJEMPLARES DE DORADA AFECTADOS POR PHD (FUENTE: : WWW.VETERINARIA.ORG Y REDVET®)

Actualmente las intervenciones que se están haciendo contra *Phd*, suelen ser preventivas o mediante vacunaciones o antibióticos. La mayoría de los lotes de Dorada son vacunados, pero ello solo les da un corto periodo de protección contra *Phd*, aunque cabe mencionar que los antibióticos ha sido uno de los primeros tratamientos a usar, pero la reducción de los mismo es prioritaria para así evitar que produzcan cepas de patógenos resistentes [4] y también reducir el impacto negativo que ello produce sobre el consumidor [5] y no muchos menos importante, sobre el medio ambiente [5-6].

El sistema inmunológico se define como un sistema capaz de proteger al organismo, contra agentes que puedan causar enfermedad o infección [7]. El sistema de los peces se basa en respuestas tanto inmunitarias adaptativas como adquiridas [8]. El sistema

inmunológico innato se caracteriza por una protección del individuo sin ningún tipo de exposición al patógeno, ya que actúa como primera línea de defensa hacia agente extraños, hasta la activación del sistema específico (Figura 4). En cambio, el sistema especifico se activa por la respuesta de un patógeno invasor y tras una exposición repetida del mismo [9].

El sistema inmune innato tiene una gran importancia en los peces, ya que es el primero en enviar la señal para proteger al individuo de la infección, que a su vez conecta con una gran variedad de mecanismos, que parece ser más importante en los peces que en los vertebrados superiores [10]. Constituido por factores físicos (escamas, piel, branquias y barreras mucosas en general), factores humorales (receptores asociados a células y moléculas solubles de plasma y otros fluidos corporales, como el complemento sistema, los anticuerpos naturales, NAbs, citocinas / quimiocinas) y células (células fagocíticas, células citotóxicas inespecíficas, células epiteliales y dendríticas).

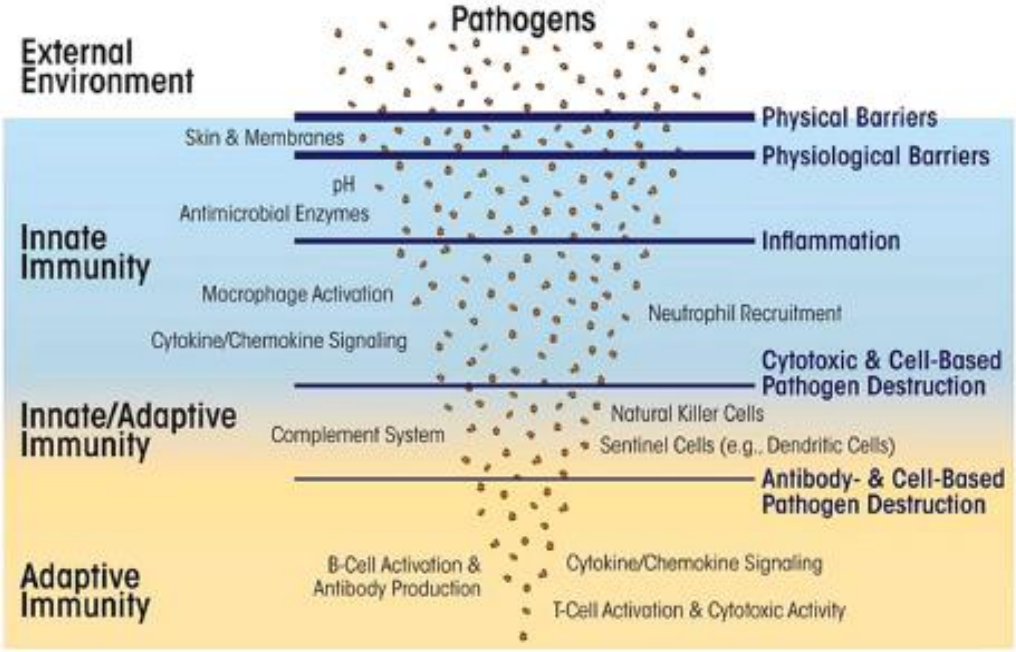


FIGURE 4. SISTEMA INMUNITARIO DE LOS PECES (SPIERING, 2015)

En cuanto a las barreras físicas de defensa de los peces, ya que se encuentra constantemente sumergidos en un medio acuático, y por consiguiente están en contacto con posibles patógenos u otros agentes nocivos, conociéndose como la primera barreara de defensa del sistema inmunológico innato son las escamas, las células epiteliales que recubre la piel, las branquias, el tracto digestivo y el moco que los envuelve [11-12]. En cuanto al moco que lo envuelve, contiene una amplia gama de sustancias inmunitarias, como, lectinas, lisozima, peroxidasa, antibacterianos e IgM entre otros, que inhiben la entrada o incluso la eliminación de patógenos [13].

La respuesta celular se define mediante la barrera física y las células especializadas, que son capaces de matar y digerir patógenos si estos últimos rompen las barreras físicas [14]. Las células sistema inmunológico innato de los teleósteos son células fagocitas, que reconocen y eliminan a patógenos invasores y otras moléculas de tejidos dañados [12].

La respuesta humoral en los peces teleósteos se compone de un gran número de sustancia de defensa inespecífica, como inhibidores del crecimiento, diversas enzimas líticas y componentes de las vías del complemento, principalmente lectinas, anticuerpos naturales, citocinas, quimiocinas y péptidos antibacterianos [11].

La peroxidasa se conoce como una de las enzimas más importante, utiliza peróxido de hidrógeno y produce iones para formar cloruros y cloraminas, siendo estos unos agentes microbicidas muy tóxicos e importantes en una vía de defensa inmunitaria conocida como estallido respiratorio [15]. La peroxidasa, que se encuentra en el suero, pero también en el moco, considerándose esencial para la inmunidad de la mucosa y la defensa de la piel [16].

Las proteasas, que a menudo se les conoce por ser un grupo que se encarga de la degradación de patógenos u otras sustancias, además también actúan activando y aumentado la producción de algunos otros componentes inmunológicos, como las inmunoglobulinas y los péptidos antimicrobianos [17].

Una vez definido en sistema inmunológico de los teleósteos vamos a definir dos conceptos que nos interesan en nuestro estudio, como sería el concepto de resistencia y tolerancia hacia una infección.

La resistencia a las enfermedades se define como la capacidad de resistir y controlar la infección, ya que todos los animales son susceptibles a la infección inicial, pero difieren en su capacidad para obstaculizar la entrada de patógenos, su replicación, liberación y supervivencia. Se puede medir como el nivel de carga de patógenos dentro de un animal (recuento de células virales o bacterianas) para un nivel dado de exposición: en la Figura 5a, los mismos niveles de exposición dan como resultado una carga de patógenos más alta para el animal B que para el animal. A, por lo que A puede considerarse más resistente que B.

La tolerancia y la resiliencia a las enfermedades reflejan las relaciones entre el rendimiento productivo y la carga de patógenos o el nivel de exposición y se vuelven importantes en un contexto de producción animal.

La tolerancia es la capacidad del huésped para limitar la influencia en la salud causada por una infección por patógenos; se puede ver como la relación entre carga de patógenos y desempeño productivo (Figura 5b): cuando la carga de patógenos aumenta, el desempeño productivo de los animales con niveles más altos de tolerancia (animal B en la Figura 5b) se deteriora menos que el de los animales menos tolerantes (animal A en la Figura 5b).

La resiliencia (o solidez) es la capacidad de mantener la productividad al hacer frente a diferentes desafíos ambientales. Puede evaluarse como el desempeño productivo exhibido a un nivel dado de exposición y la capacidad de limitar formas crónicas de la enfermedad: en la Figura 5c, B es más productivo que A cuando no existe exposición, pero A muestra mejores desempeños productivos que B cuando el nivel de exposición supera un umbral dado, lo que lleva a la conclusión de que A es más resistente que B [18].

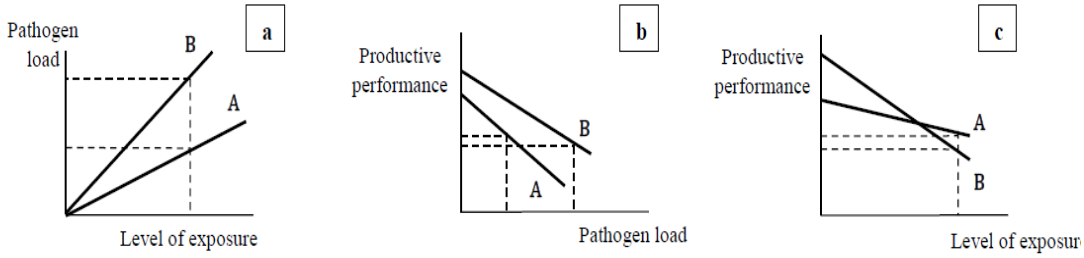


FIGURE 5. RELACIÓN ENTRE LOS NIVELES DE EXPOSICIÓN Y CARGA DEL PATÓGENO. A) CARGA DEL PATÓGENO Y EVOLUCIÓN DE LA PRODUCCIÓN. B) NIVELES DE EXPOSICIÓN Y EVOLUCIÓN DE LA PRODUCCIÓN. C) DEFINIR LA RESISTENCIA, TOLERANCIA Y RESILIENCIA, COMO NOS MOSTRÓ DEKKER (2016).

Para comprobar si un pez es resistente, las pruebas de desafío son las mas útiles, donde los peces se someten a una infección controlada, en un ambiente estándar y utilizando un solo patógeno, en ese momento. Estas características de los desafíos, resultan en una maximización de la reproductibilidad del procedimiento y mejoren los datos en términos de tolerancia del individuo al patógeno. Por lo que, los peces que son utilizados en el desafío, nunca podrán ser usados como futuros reproductores, debido a cuestiones sanitarias. Para realizar una estima de parámetros genéticos se utilizarán la información de los parentales y por esta razón es preciso conocer la relación entre los individuos. Una de las vías para llegar a ello, es el uso de marcadores moleculares como los microsatélites, que nos permite realizar la construcción del pedigrí y la detección de la endogamia. De igual modo el uso de PCRs multiplex, permite a los genetistas el realizar múltiples PCR reduciendo su coste económico por reacción. Desde hace algunos años se vienen desarrollando varias PCR multiplex en dorada (*Sparus aurata* L.) [19-24].

No podemos olvidar que dicho estudio se encuentra enmarcado dentro de un programa mejora de la competitividad del sector de la dorada a través de la selección genética (PROGENSA®), hasta el momento la selección genética se está haciendo a favor del crecimiento y en contra de malformaciones esqueléticas. En este sentido, nos planteamos estudiar la variación genética para la resistencia a *Photbacterium damselae* subespecie *pisccicida* como posible futuro de criterio de selección.

Además, una de las mediciones útiles podría ser los marcadores inmunológicos para la detección de animales resistentes; son principalmente componentes del sistema inmunológico innato o no específico, como la actividad antimicrobiana (actividades bactericida y peroxidasa) y los niveles de inmunoglobulinas IgM.

El objeto de estudio fue valorar los parámetros genéticos por primera vez en dorada (*Sparus aura* L.) para la resistencia a *Phd* mediante marcadores inmunológicos (actividad peroxidasa y actividad bactericida, nivel de inmunoglobulina M), calculando las heredabilidades de ellos con respecto al peso corporal del animal, para con ello conseguir un programa de selección de conducido por los peces del estudio.

OBJETIVOS

El objetivo principal es estimar los parámetros genéticos de resistencia al patógeno *Photobacterium damselae* subspecies *piscicida*.

Para ello, se desarrollan objetivos específicos que son los siguientes:

- Caracterización fenotípica de las poblaciones estudiadas para las variables de peso, resistencia a Phb y marcadores inmunológicos

- Estimación de la varianza genética del carácter resistencia a Phb y los marcadores inmunológicos innatos, así como sus correlaciones genéticas entre estos y con el peso de la dorada (*Sparus aurata* L.)

PAPER

Genetic parameters for *Photobacterium damselae* resistance, immunological markers and body weight in Gilthead seabream (*Sparus aurata*)

Antonio Vallecillos[1], Rubén Perez[1], Elena Chaves-Pozo[2], Marta Arizcun[2], Juan M. Afonso[3], Concepción Berbel[4], Jaume Pérez-Sánchez[5], Emilio María-Dolores[1], Eva Armero[1]*.

[1] Department of Agronomic Engineering, Technical University of Cartagena, Paseo Alfonso XIII 48, Cartagena, Spain
[2] Spanish Institute of Oceanography, Oceanographic Center of Murcia, carretera de la Azohía s/n, Puerto de Mazarrón, Murcia, España.
[3] Institute of Sustainable Aquaculture and Marine Ecosystems (GIA-ECOAQUA), Carretera de Taliarte S/N, 35214 Telde, Las Palmas, España
[4] Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) Centro El Toruño, Junta de Andalucía, Camino Tiro Pichón s/n, 11500 El Puerto de Santa María, Cádiz, Spain.
[5] Nutrigenomics and Fish Growth Endocrinology, Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, España.
* To whom correspondence should be addressed: Tel: +34-968-325538; Fax: +34-968-325433. E-mail: eva.armero@upct.es

Simple Summary: Most breeding programs for gilthead seabream (*Sparus aurata* L.) are focusing on improve growth performances and morphology traits, later other objectives (feed efficiency and product quality) have been established. The aim of this work is to estimate genetic parameters for *Photobacterium damselae* subsp. *piscicida* (*Phd*) resistance, *humoral immune markers* and their correlation between them and with the *body weight* in order to be considered in the establishment of a selective breeding program

Abstract: A challenge test for *Photobacterium damselae* subsp. *piscicida* (*Phd*) resistance in two juvenile populations of gilthead sea bream (*Sparus aurata* L.): F2_ATL and F0_MED was carried out. At 272 days post-hatching, fish were sampled for weight and a plasma sample was collected to measure *humoral immune markers* (*peroxidase activity*, *bactericidal activity* and *IgM immunoglobulins levels*), and after fish were inoculated with bacteria *Phd*. From this time, survival fish were recorded for 9 days, and days to death was registered. Heritabilities for *body weight* and for *Phd resistance* were moderate, however for *days to death* was low. Regarding *humoral immune markers*, for *peroxidase activity* was moderate, and for *IgM levels* and for *bactericidal activity* was low. Genetic correlations for *body weight* with *Phd resistance* and *days to death* were high and positive, while with *peroxidase activity* and *IgM levels* tended to be positive

but these estimates were not accurate. Regarding genetic correlations between *Phd resistance* and *humoral immune markers* 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* or absence of deformities, are proposed to be included in a selective breeding program to raise fish that are capable of coping with diseases and environmental challenges.

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

1. Introduction

The gilthead seabream is a one of the most important species in the Mediterranean area for fisheries and aquaculture, with a production of 252,406 tons through aquaculture in Europa and rest of the Mediterranean countries. The three most important countries to produce gilthead seabream were Greece, Turkey and Spain, in this order. Spain produces 13,521 tons, which represented up to 6% of world production [1]. Disease outbreaks are serious threats that cause important economic loss. The farming activity and the open design of Mediterranean aquaculture systems allow the transmission of infectious pathogens within and among farm facilities [2]. The main pathogenic microorganisms isolated affecting gilthead seabream 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. damselae* subsp. *piscicida* cause epizootics with highest degree of mortalities resulting in severe losses for the fish farming industry [3].

Photobacterium damselae subspecies *piscicida* (Phd) was first described in wild populations of white perch and striped bass, but the natural hosts of the pathogen are currently a wide variety of marine fish. Phd caused the “pseudotuberculosis” that is recognized as granulomatous-like lesions n the spleen and kidney of affected fish. External symptoms are weight loss, a darkening of the skin, localised necrosis in the gills, and distended stomach, and internally are tumours of between 0.3-0.5 mm in some organs and a congestion and dilatation of the digestive tract [4]. This 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 the gonadal fluids, as well as horizontal through the water route, by this route the bacteria is able to infect its host through the gills, the digestive system and the skin [5]. The peak of proliferation of said pathogen occurs in summer, with high temperatures. Common interventions to prevent or control this disease are vaccines and antibiotics. Most batches of gilthead seabream are vaccinated, but protection does not last for long period. Antibiotics have been the first treatment to control photobacteriosis outbreaks, however reducing the use of antibiotics is a priority in order to avoid the development of resistant pathogen strains [6] and to reduce the negative impact of drug residues on consumer's health [7] and the environment [8-9].

Therefore, selective breeding programs for disease resistance play a key role in hindering the spread of pathogens, due to a long-term control of the disease [10] and because genetic improvement is cumulative and permanent [11]. In addition, the improvement in disease resistance inherited by new generations may provide protection at the larval stage, when typically, the immune system is not completely developed [12].

Selective breeding programs have been initiated in gilthead seabream to improve growth performances and morphology traits, later other objectives (feed efficiency and product quality) have been stablished [13], and currently two breeding programs for disease resistance are supported [14].

Disease resistance can be defined as the ability to resist and control the 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 [15]. To investigate if a fish is resistant challenge tests are the most used, where fish are subjected to a controlled infection, in a standardized environment, using a pathogen to the time. These characteristics minimize variation due to uncontrolled sources, maximize the reproducibility of the procedure, and improve the interpretation of data in terms of individual tolerance to the pathogen. Infected fish cannot be chosen as future breeders due to sanitary reasons. To estimate genetic merit of breeding candidates, the information of their relatives is used and for this reason the first step in a breeding program is to know the relationship between individuals. The use of molecular markers such as microsatellites allows pedigree reconstructions and the detection and avoidance of inbreeding. Concurrently, the development of multiplex PCRs allows geneticists to

reduce the economic cost per reaction. Several multiplex PCRs have been described for gilthead seabream; [16-18]; and more recently [19-21].

In addition, useful markers to identify 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 *IgM immunoglobulins levels*. In fish, the innate immune response has been considered an essential component in combating disease incidents [22]. Teleost fish, in general, have *IgM levels* as their immunoglobulin and are capable of eliciting effective specific humoral antibody responses against various antigens. Peroxidase is an important enzyme that uses hydrogen peroxide and produces ions to form chlorides and chloramines that are also highly toxic and important microbicidal agents in an immune defense pathway known as respiratory burst [23].

The aim of this work was to estimate genetic parameters, first time in gilthead seabream, for *Phd resistance* along with *humoral immune markers* at naïve conditions (*peroxidase activity* and *bactericidal activity*, *immunoglobulin M level*), and their correlations between them and with the *body weight* in order to be considered in the establishment of a selective breeding program conducted by the own fish data.

2. Materials and Methods

To ensure that animal welfare standards are maintained, anesthetic was used within the sampling procedure. All animal experiments described in this manuscript fully comply the recommendation 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 "Región de Murcia", Spain (approval number A13191103). Whenever necessary, fish were anesthetized with 20 µL of clove oil/mL of sea water.

2.1. Animals

The experiment was carried out in gilthead seabream juveniles, which were obtained from two different broodstock, belonging to the Spanish genetic breeding program PROGENSA®. The first broodstock (n = 133, 57♂ and 76♀) coming from Mediterranean Sea was maintained in Instituto Español de Oceanografía, Mazarrón, Murcia (IEO), and never was subjected to genetic selection (F0 generation), hereinafter this broodstock is called F0_MED. The second broodstock (n=51, 17♂ and 34♀) originally came from Andalusian coast Atlantic Ocean (ATL) was maintained in Instituto de Investigación y Formación Agraria y Pesquera, el Toruño, Puerto de Santa María, Andalusia (IFAPA), and they were the second generation (F2) under genetic selection to increase weight at harvest and to reduce deformities, this

broodstock is called F2_ATL. The female/male ratio was approximately 2:1 in the tanks, were under a controlled photoperiod (8L:16D) to synchronize maturation and egg release was initiated at the beginning of December 2016. During this period, animals were fed *ad libitum* by Vitalis Cal (Skretting), and egg production was monitored daily. When total egg production became stable, two egg batches were established: one at the end of February and another in early April 2017. In both cases, eggs from broodstocks were collected and pooled for 4 consecutive days (4DL model) to maximize family production. Incubation was carried out in cylinder conical tanks (1000 L) at a density of 500–1000 larvae L^{-1}. Water conditions were as follows: Temperature 19.0 ºC, salinity 34‰ and dissolved oxygen 6.4 mg. L^{-1}. So, 337 offspring of F0_MED and 271 offspring of F2_ATL were used, the fingerlings 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*. [24].

2.2. Bacteria culture

The *Photobacterium damselae* subsp. *piscicida* (*Phd* 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 steril phosphate buffered saline (PBS) by centrifugation at 2,700xg for 15 minutes at 4ºC. The optical density of the bacterial suspensions was measured at 540 nm, and the number of colony forming units (u.f.c.) / ml was calculated with a growth standard curve. The bacterial suspension was adjusted to $8x10^5$ bacteria/ml.

2.3. Challenge test

Previous to the infection, fish from both populations F0_MED (n= 400) and F2_ATL (n=360) 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. The blood obtained were allowed to clot at 4ºC for 16 hours and centrifuged at 10,000xg for 10 minutes at 4ºC to obtain the serum that was frozen at -80ºC until further analysis. 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 losses due to handling. Even so, a 23% average mortality was recorded in the days after extraction, being slightly higher among the F2_ATL fish. The fish were left to recover under an open circuit culture conditions for at least 20 days.

Then, fish were transport to the infection facilities, randomly distributed between 5 rectangular tanks of 200 L capacity, provided with an independent recirculation system composed of a mechanical and biological filter, two aerators, a stabilization tank and a submersible recirculation pump, and intraperitoneally injected (i.p.) with 100 µL of PBS containing a sublethal dose of Phd ($8x10^4$ bacteria) at 272 days post-hatching (dph). A

control groups injected with PBS alone was also performed in a similar tank and independent recirculation system. Mortalities were recorded twice a day for the first 3 days and daily from day 4 onwards. After 9 days of i.p., the fish overcame the infection as the cumulative mortality in the last three days was less than 3 fish in all tanks. All the survivor fish were sacrificed using an excess of anesthesia (40 µL/L of clove oil in seawater).

2.4. Recorded traits

2.4.1. Body weight

Before inoculation, *body weight* (*BW*) was measured thanks to a scale with an accuracy of 0.1 g and a portion of the tail fin was stored in ethanol.

2.4.2. Resistance disease and days to death

Regarding resistance to infection, survivor fish were assigned the number 0 and the susceptible ones the number 1. *Mortality rate* was calculated as number of dead fish divided into the total fish within 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 9 days. The infection was considered finished after 9 days, because the number of deaths accumulated in the last three days was less than 3 in all tanks.

2.4.3. 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 markers of adaptative humoral immune status in serum samples.

- *Peroxidase activity*

The peroxidase activity levels in serum were measured according to a protocol previously described [25]. 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 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 on an orbital incubator at 200-250 rpm and adjusted to 10^8 bacteria / mL with TSB. The absorbance of bacteria cell cultures was measured at 600 nm and used to know the concentration based on growth curves.

The antibacterial activity of serum was determined by evaluating their effects on the bacterial growth of Vh curves using a method previously described [26]. 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 during 2 h at room temperature. Then, 150 µL of TSB were added and the absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25 °C. Samples without 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 % of bacterial growth inhibition per mL of plasma.

- Total levels of inmunoglobulins M

A direct ELISA was used to detect total IgM in gilthead sea bream serum. For IgM detection, MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) 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 sea bream IgM [27] at the optimal dilution of 1:100 in PBS with 1% BSA were added, and plates were incubated for 1 h at RT. After washed 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 were 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. After 10 min of incubation at RT, the reaction was stopped 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).

2.5. Microsatellite Genotyping and parental assignment

The broodstock and offspring were genetically characterized, for this purpose, the DNA was extracted from the caudal fin, it was conserved in absolute ethanol at room temperature using the *DNeasy kit* (QIAGEN®) and then kept at 4ºC. Next, DNA quantity and quality were determined to 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 [28] for genotyping of broodstock and offspring. The electropherogram was analyzed by using to Microsatellite analysis cloud (Thermo Fisher Scientific). Direct count heterozygosity in the offspring of each population was calculated with Excel package called Gene Alex [29]. For the parental assignment the exclusion method as implemented in VITASSING (v.8_2.1) software [30], was used.

2.6. Statistical analysis

All data were tested for normality and homogeneity of variances using SPSS (v.25.0) [31] and phenotypical data for each trait were analyzed by the General Linear Model (GLM):

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

in which Y_{ij} is an observation of an individual j from the origin i, μ is the overall mean, origin is the effect of the broodstock origin (i= F0_MED or F2_ATL), b (not included for BW) is the regression coefficient between the analyzed variable and the covariate *BW*, and e_{ij} is a random residual error. The level of significant difference was set at $P < 0.05$. Resistance to infection was analysed by Chi^2 (nonparametric tests) using the SPSS® (v.25.0) to detect the effects of the broodstock origin.

Genetic parameters were estimated under a Bayesian approach by 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* the fixed tank effect, *u* the random animal effect, covariate *body weight* was included and *e* the error. Performed by using gibbs1f90 program for all traits except that for resistance disease that was considered a threshold trait and analyses with the program thrgibbs1f90 developed by Misztal *et al*.

[32]. The analysis was carried out between two traits each time. The following multivariate normal distributions were assumed *a priori* for random effects:

$$P(\beta) \sim k;$$
$$P(u|G) \sim (0; G \otimes A);$$
$$P(e|R) \sim (0; R \otimes A);$$

(3)

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

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

(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 5 iterations. Density plots to represent posterior marginal distribution of heritabilities, posterior means (PM) and the interval of 95% of the highest posterior density (HPD 95%) were obtained through R Development Core Team [33].

3. Results

3.1. Phenotyping

Phenotypic data for BW and immunological markers in gilthead sea bream juveniles (272 dph) from the two broodstocks are shown in table 1. In addition, BW distribution was further analyzed in depth since F2_ATL broodstock has been subjected to selection for two generations to improve body weight (Figure 6). 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 23.2 g) than F0_MED (70 % of fish were in the range 9.8 to 16.8 g). The weight for the 30% of the heaviest fish was over 19.2 g for F2_ATL and 14.4 g for F0_MED. This threshold value was closer to their respective average for F0_MED due to its data was more clustered.

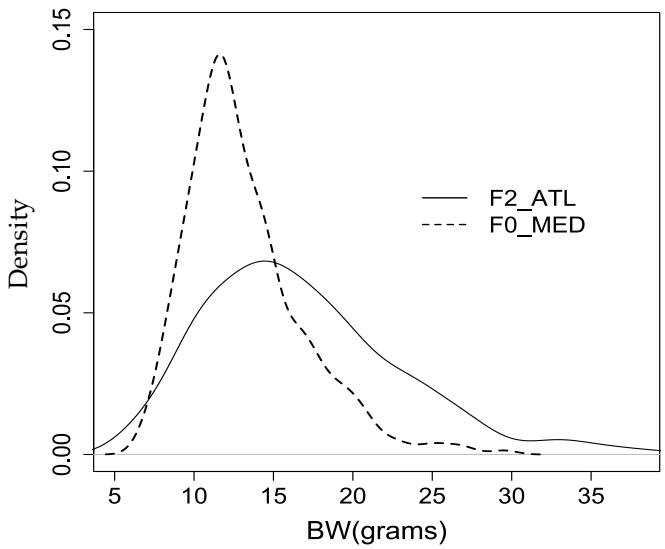
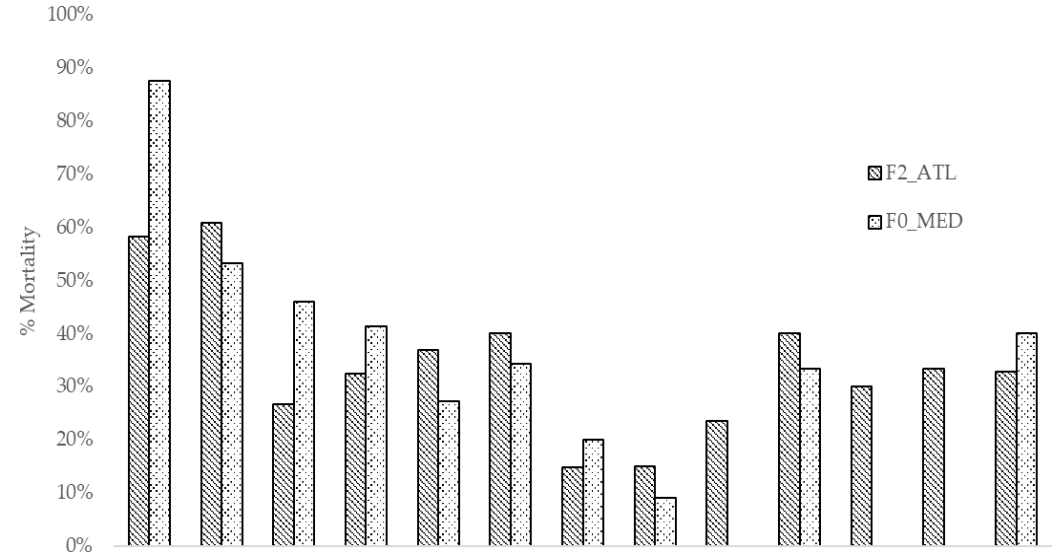


FIGURE 6. BODY WEIGHT (BW) DISTRIBUTION FOR BOTH POPULATIONS: F2_ATL = OFFSPRING FROM BROODSTOCK ORIGINALLY FROM THE ATLANTIC OCEAN AND THEY WERE THE SECOND GENERATION (F2) UNDER GENETIC SELECTION TO INCREASE WEIGHT AT HARVEST AND TO REDUCE DEFORMITIES (N = 271), AND F0_MED = OFFSPRING FROM BROODSTOCK FROM MEDITERRANEAN SEA THAT NEVER HAS BEEN SUBJECTED TO GENETIC SELECTION (N = 337).

Mortality rate was calculated overall and depending on the weight for both populations (Figure 7). No population effect was observed on *mortality rate* (χ^2 =0.15 with one freedom degree). Overall *mortality rates* were 32.8% and 40% in juveniles from F2_ATL and F0_MED, respectively. When *mortality rate* was analyzed depending on the *BW*, the highest *mortality rate* occurred for weight from 6 to 10 g. In F0_MED juveniles mortality tended to decrease with weight gain, except for 24-25.99 g range in which the percentage was high (33.3%) but it was calculated with only three fish. However, in F2_ATL juveniles' mortality was more equally distributed along the *BW* ranges, with the lowest value from 18 to 21.99 g range, although over 18 g there are few fish.



BW range	1	2	3	4	5	6	7	8	9	10	11	12	Overall
F2_ATL	7/12	14/23	8/30	12/37	14/38	14/35	4/27	3/20	4/17	4/10	3/10	1/3	
F0_MED	7/8	24/45	45/98	31/75	12/44	11/32	3/15	1/11	0/3	1/3	0/2	0/1	
Days to death	4.15 (0.73)	5.22 (0.40)	6.09 (0.29)	6.45 (0.31)	6.99 (0.36)	6.49 (0.40)	7.86 (0.50)	8.10 (0.59)	6.46 (0.90)	7.50 (0.94)	7.25 (1.63)	9.00 (3.26)	

FIGURE 7. MORTALITY RATE UPON INFECTION WITH 5X104 PHD/FISH 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), 9 (22.00-23.99G), 10 (24.00-25.99G), 11 (26.00-27.99G), 12 (28.00-29.99G). IN THE TABLE IS REPRESENTED THE NUMBER OF DEATH FISH DIVIDED INTO THE TOTAL NUMBER OF FISH AND DAYS TO DEATH (MEAN AND STANDARD ERROR IN BRACKETS), IN EACH BW RANGE. F2_ATL = OFFSPRING OF BROODSTOCK ORIGINALLY FROM THE ATLANTIC OCEAN AND THEY WERE THE SECOND GENERATION (F2) UNDER GENETIC SELECTION TO INCREASE WEIGHT AT HARVEST AND TO REDUCE DEFORMITIES (N = 271), AND F0_MED = OFFSPRING OF BROODSTOCK FROM MEDITERRANEAN SEA THAT NEVER HAS BEEN SUBJECTED TO GENETIC SELECTION (N = 337).

In addition, disease resistance was daily measured and *days to death* were shown (Table 1). The average *days to death* was 6.5 (standard error or s.e. = 0.2), and no significant differences were observed between populations. However, the *BW* has positive effect on *days to death* (regression coefficient = 0.134 days/g, s.e. = 0.027), the heavier fish died in the last days of the experiment. In general, it was observed that heavier fish needed more days to death (Figure 7).

Regarding *humoral immune markers*, juveniles from F2_ATL showed much higher, more than twice, *peroxidase activity* and slightly higher *IgM levels* than those from F0_MED. For *bactericidal activity* no significant differences were observed. The *BW* showed negative effect for *peroxidase activity*, but only in F0_MED juveniles, and positive effect for *bactericidal activity*, since the more *BW*, the more levels of *bactericidal activity*.

Table 1. Phenotypic results (least square means ± standard error) for body weight and *immunological markers* for gilthead seabream juvenile from two populations at 272 days post-hatching

Broodstocks origin[1]	**F2_ATL**			**F0_MED**			**Cov *BW***	
	n	**LSM**	**S.E.**	**n**	**LSM**	**S.E.**	**b**	**S.E**
BW (g)	271	16.8[a]	0.30	337	13.2[b]	0.27	-	-
Days to death	271	6.54	0.20	337	6.51	0.18	0.134*	0.027
Peroxidase activity (U.I./ml)	236	38.3[a]	1.78	329	15.5[b]	1.49	-0.554*&	0.227
Bactericidal activity (%)	120	18.0	1.08	268	19.2	0.70	0.063	0.122
IgM levels (O.D. 450 nm)	167	0.29[a]	0.007	291	0.26[b]	0.005	-0.001	0.001

[1] Broodstocks origin: F2_ATL = offspring from broodstock originally from the Atlantic Ocean and they were the second generation (F2) under genetic selection to increase weight at harvest and to reduce deformities, and F0_MED = offspring from broodstock from Mediterranean Sea that never has been subjected to genetic selection; *BW = body weight*; *IgM levels= Immunoglobulins M*; & = It was only significant for F0_MED origin; ab: different superscripts within each row indicate significant differences between origins ($P < 0.05$); *IgM*, *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 ($P < 0.05$), b = regression coefficient for *BW*

3.2. Microsatellite Genotyping and parental assignment

For F2_ATL, 87% of the offspring was assigned, of which 83.9% was assigned to a single couple of parents. In the case of F0_MED, it was obtained a 97.54% parental assignment, of which a 92% was assigned to a single couple. After the assignment, unequal breeder contribution was observed. In F2_ATL, 9 out of 34 females produced 55% of the offspring but all of the females contributed to the offspring, and 2 out of 17 males contributed with 49% of the offspring and one male did not contribute. Similarly, in F0_MED, 8 out of 76 females contributed with 46% of the offspring and 19 females did not produce any offspring, and 12 out of 57 males contributed with 54% of the offspring and 9 males did not contribute.

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

3.3. Genetic parameters

3.3.1. Heritability

Heritability for *BW* at 272 dph was moderate (PM=0.20 and HPD = [0.08-0.30]). For *Phd resistance* heritability was moderate (0.32 [0.15-0.45]), however for *days to death*

was low (0.05 [0.02-0.14]). Regarding *humoral immune markers*, for *peroxidase activity* was moderate 0.30 [0.16–0.48], and for *IgM levels* and *bactericidal activity* was low (0.10 [0.01–0.23] and 0.09 [0.00-0.19], respectively) (Figure 8).

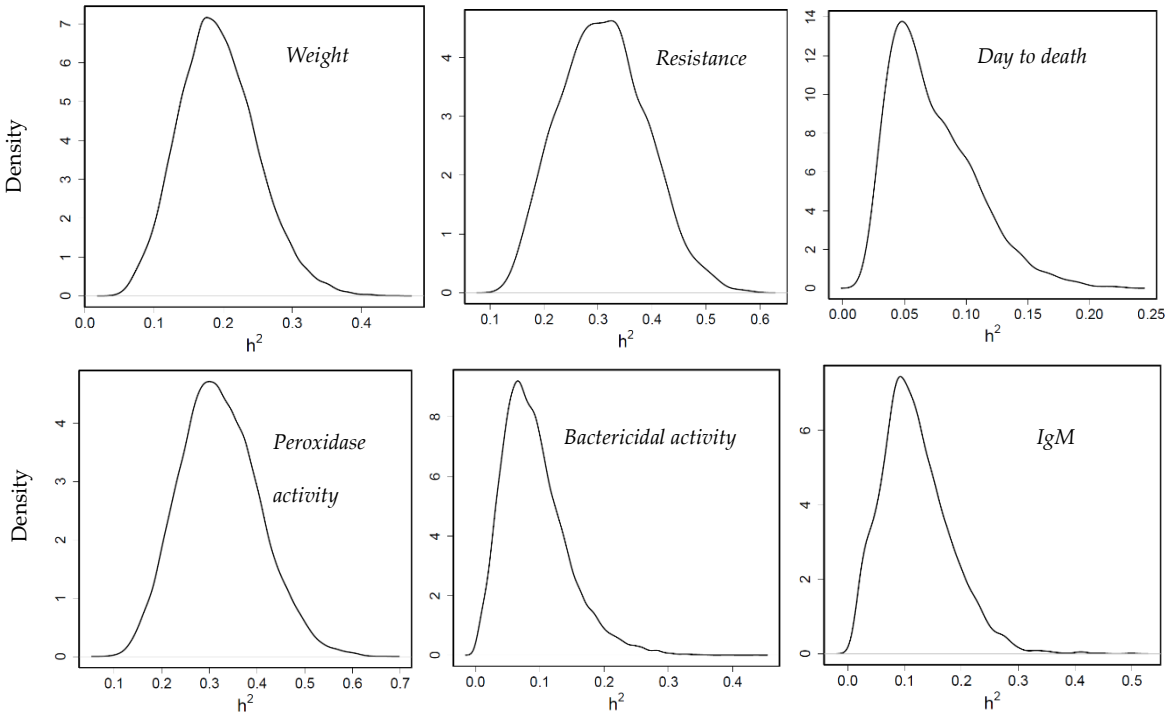


FIGURE 8. POSTERIOR MARGINAL DISTRIBUTION OF HERITABILITIES OF THE HUMORAL IMMUNE MARKERS ANALYZED IN THIS WORK, WEIGHT, TOLERANCE OF SUSCEPTIBILITY TO THE PATHOGEN AND DAYS TO DEATH ESTIMATED FROM 609 GILTHEAD SEA BREAM AT 272 DPH. H2=HERITABILITY.

3.3.2. Genetic correlations

Weight showed very high (close to 1) and positive genetic correlation with *Phd resistance* and *days to death*. Genetic In consequence, *Phd resistance* and *days to death* were highly and positively correlated. Genetic correlation between weight and *peroxidase activity* and *IgM levels* tended to be positive but the estimate was not accurate. On the contrary, genetic correlation between *weight* and *bactericidal activity* was high but negative (close to -1) (Figure 9). For *humoral immune markers* there were less data, especially for *IgM levels* and *bactericidal activity*, therefore genetic correlations should be considered with caution.

Genetic correlations between *Phd resistance* 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*, however correlation between *days to death* and *IgM levels* was less accurate and positive. *Peroxidase activity* and *IgM levels* were negatively correlated.

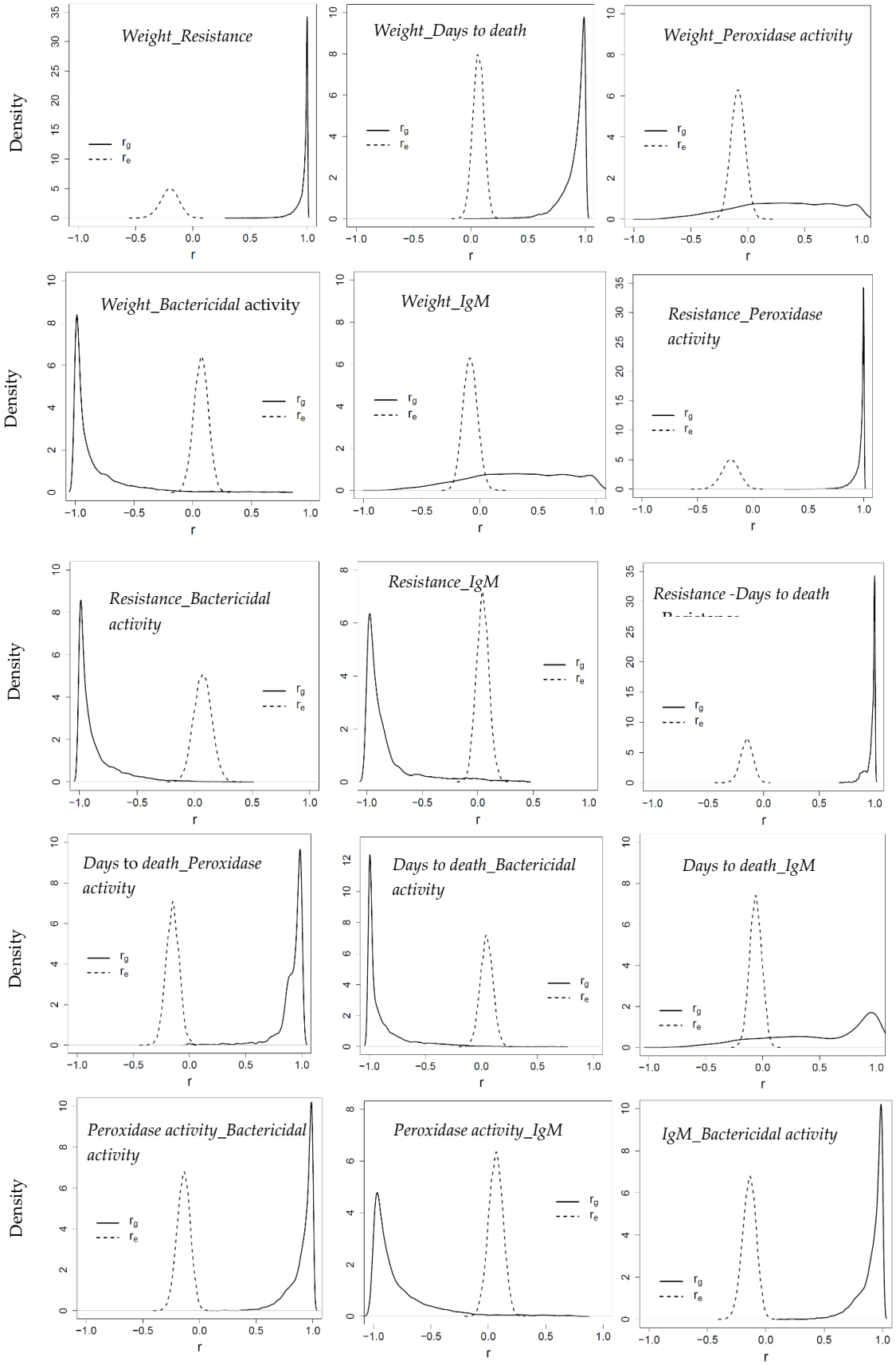


FIGURE 9. POSTERIOR MARGINAL DISTRIBUTION OF GENETICS CORRELATIONS OF BW, PHOTOBACTERIUM DAMSELAE RESISTANCE, DAYS TO DEATH AND IMMUNOLOGICAL MARKERS (PEROXIDASE ACTIVITY WITH 609 DATA, BACTERICIDAL ACTIVITY WITH 457 DATA AND IMMUNOGLOBULINS M (IGM) WITH 566 DATA) GILTHEAD SEABREAM AT 272 DPH. RG = GENETIC CORRELATION; RE = RESIDUAL CORRELATION.

4. Discussion

F2_ATL population was the offspring of the second generation of breeders from PROGENSA project (PROGENSA®, http://www.progensa.eu) selected to increase growth rate and to decrease deformities, while F0_MED population came from a new broodstock from Southern Mediterranean Sea that never has been subjected to selection. At the beginning of PROGENSA project (2009 year), zero generation, this Atlantic (F0_ATL) population was studied together other Northern Mediterranean Sea Population. At that time [34], higher *BW* for the Mediterranean populations than the Atlantic Ocean population was observed, contrary to current work. It is difficult to analyze the selection response for growth of ATL population since *weight* was measured at different ages and rearing conditions were not the same. When these populations were genotyped for SMSa1 multiplex, no effect of population or selection process were observed when F2_ATL was compared with F0_MED heterocigosity (Ho = 0.72), revealing that there was no inbreeding danger by the moment. Heritability for *BW* (0.2) was in the range of other authors. [35] Carballo *et al*. showed *BW* heritability around 0.18 and 0.06 in two batches with animals at 80 dph and at 140 dph, respectively. As in García-Celdrán *et al.* [34] a heritability of 0.11 and 0.25 at 163 and 690 dph respectively was obtained, and they pointed out that heritability estimates for growth traits increased with age when they compared juveniles with commercial size fish.

Regarding *disease resistance*, no population effect was revealed on *mortality rate* or *days to death* in accordance with Antonello *et al.* [36], when studied *Phd resistance* for different Adriatic and Atlantic broodstocks. In our work, *days to death* was around 6.5 days when the challenge was based on a dose of the $8x10^4$ bacteria / fish injection. In Antonello *et al.* [36], when fish were infected by immersion in sea water containing $1x10^5$ CFU of Phd, a first mortality peak at day 7 post challenge and a second one at day 11were shown and most fish died. About genetic variation, heritability was moderate for *resistance* on liability scale and low for *days to death*. Our results are in keeping with Antonello *et al.* [36], in which they observed lower heritability for *days of survival* post *Phd* 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 at a specific day. Palaiokostas *et al.* [37] 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. Other works that have studied heritability for other disease resistance or in other species also found moderate to high value: salmon pancreas disease virus (SPDv) in Atlantic

salmon (*Salmo salar*) [0.21 ± 0.01] [38], infection pancreas necrosis disease virus (IPNV) in Atlantic salmon (*Salmo salar*) [0.38 ± 0.02] [39], nervous necrosis virus (NNV) in Atlantic cod (*G. morhua*) [0.75 ± 0.11] [40] and in European seabass (*D. labrax*) [0.26 ± 0.11] [41]. Heritability of mortality traits are frequency dependent, with maximal values reported at intermediate mortality levels [42]. In any case, it is expected to have good response when breeders are selected through the offspring to improve *Phd resistance*.

For genetic correlations, *weight* was positive and highly correlated with *Phd resistance* and *days to death*, in accordance with Antonello *et al.* [36] when estimated correlation between *Phd resistance* and *body length*. However different results appear in other species and other diseases. On one hand, some authors, agree with our results, and observed positive correlation in infection by *lymphocystis* disease virus (LCDV) in Gilthead seabream (*Sparus aurata* L.) [35]. On the other hand, other authors showed negative correlation in viral nervous necrosis (VNN) in different species of aquatic animals [41] or Columnaris disease (CD) caused by *Flavobacterium columnare* in Rainbow trout (*Oncorhynchus mykiss*) [43].

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 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 [44]. There is a link between stress-immunodepression-disease susceptibility, thus developing lines of farmed animals highly resilient (ability to maintain productivity when coping with different environmental challenges) might be a strategy to improve disease resistance [15] or reciprocally, improving disease resistance fish could be able to cope with environmental challenges. In our study, F2_ATL showed the highest *peroxidase activity*, but less overall *mortality rate*. Therefore, the higher *peroxidase activity* is likely to make the fish better able to cope with the disease. In our knowledge there is one work to study genetic variation for *immunological markers* (Lysozyme activity, haemolytic activity, total level of *IgM*, and levels of antibodies measured after the immunization) in Atlantic salmon challenged to *Aeromonas salmonicida* and *Vibrio salmonicida*. A significant genetic variation in lysozyme activity was found, 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 [45]. In our work, *humoral immune markers* were measured before the infection, and also, we found only moderate heritability for

peroxidase activity that showed high genetic correlation with disease resistance. Thus, some *humoral immune markers* could be used in indirect selection to improve *disease resistance*, and the selection could be accomplished by the own phenotypical data. 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 raise fish that are capable of coping with diseases and environmental challenges and show good performance for productive traits, even that can enjoy better animal welfare; these proactive fish that have been named as "active coping" [46-47].

Other studies have investigated about genes controlling disease resistance. Dios *et al*. [48] investigated different gene expression in brain of gilthead seabream infected with nodavirus, and Fjalstad *et al*. [49] pointed out some advances in transgenic salmon including rainbow trout lysozyme gene. Future research should be continued in the establishment of a breeding program to improve disease resistance and performance traits, and searching for the genes or Quantitative Trait Loci (QTL) that are controlling disease resistance.

5. Conclusions

It is expecting to obtain good results in genetic breeding programs of gilthead seabream (*Sparus aurata* L.) to improve *Photobacterium damselae* resistance through a challenge test of the offspring. An alternative breeding program is suggested to select fish for their own data that consider increasing weight, reducing deformities and including humoral immune markers at naïve status, for the first time, such as increasing peroxidase activity, to lead to raise fish that are capable to deal with diseases and environmental challenges and show good performance for productive traits.

Author Contributions: E.C. and M.A. bred the animals and carried out the infection of the pathogen and collected data for further analysis; R.P. and A.V. did the genetic analysis in the laboratory; A.V. data interpretation and preparation of the manuscript; A.V. and E.A performed statistical analysis. E.A. preparation of the manuscript and supervision; J.M.A. project administration and supervision; M.M., J.P.S. and E.M.D. Supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This project has received funding from the European Maritime and Fisheries Fund (EMFF) through the PROGENSA III project (Mejora de la Competitividad del Sector de la Dorada a Través de la Selección Genética, JACUMAR program). Rubén Perez was supported by Universidad Politécnica de Cartagena Research Grant.

Acknowledgments: **To Javier Lopez Ales for his support in the laboratory work.**

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

6. References

1. Fish marine aquaculture in Spain. APROMAR. **2020**. (http://www.apromar.es)
2. Arechavala-Lopez, P.; Sanchez-Jerez, P.; Bayle-Sempere, J. T.; Uglem, I.; Mladineo, I. Reared fish, farmed escapees and wild fish stocks a triangle of pathogen transmission of concern to Mediterranean aquaculture management. *Aquacult Environ Interact*. **2013**, 3, 153-161.
3. Balebona, M.C.; Zorrilla, I.; Moriñgo, M.A.; Borrego, J.J. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Aquaculture*. **1998**, 166, 19-35.
4. Magariños, B.; Toranzo, A.E.; Romalde, J.L.; Phenotypic and pathobiological characteristics of *Pasteurella piscicida. Fish Dis.* **1996**, 6, 41-64.
5. Borrego, J.J.; Labella, A.M.; Castro, D.; Ortiz-Delgado, J.B.; Sarasquete, C. Updated of the Pathologies Affecting Cultured Gilthead seabream, *Sparus aurata. Ann Aquac Res*. **2017**, 4(1), 1033.
6. Defoirdt, T.; Sorgeloos, P.; Bossier, P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*. **2011**, 14, 251–258.
7. Heuer, O.L.; Kruse, H.; Grave, K.; Collignon, P.; Karunasagar, I.; Angulo, F.J. Human Health Consequences of Use of Antimicrobial Agents in Aquaculture. *Food safety*. **2009**, 49, 1248–53.
8. Kumar, R.R.; Lee, J.T.; Cho, J.Y. Fate, Occurrence, and Toxicity of Veterinary Antibiotics in Environment. *J Korean Soc Appl Biol Chem*. **2012**, 55, 701−709.
9. Kovalakova, P.; Cizmas, L.; McDonald, T.J.; Marsalek, B.; Feng, M.; Sharma, V.K. Occurrence and toxicity of antibiotics in the aquatic environment. *Chemosphere*. **2020**, 251, 126351.
10. Das, S.; Sahoo, P.K. Markers for selection of disease resistance in fish. *Aquacult Int*. **2014**, 22, 1793–1812.
11. Doan, Q.K.; Vandeputte, M.; Chatain, B.; Morin, T.; Allal, F. Viral encephalopathy and retinopathy in aquaculture. *J Fish Diseases*. **2017**, 40, 717–742.
12. Zapata, A.; Diez, B.; Cejalvo, T.; Gutiérrez-de Frías, C.; Cortés, A. Ontogeny of the immune system of fish. *Fish Shellfish Immunol*. **2006**, 20, 126-136.
13. Perera, E.; Simó-Mirabet, P.; Shin, H.S.; Rosell-Moll, E.; Naya-Catalá, F.; De las Heras, V.; Martos-Sitcha, J.A.;Karalazos, V.; Armero, E.; Arizcune, M.; Chaves, E.; Berbel, C.; Manchado, M.; Afonso, J.M.; Calduch-Giner, J.; Pérez-Sánchez, J. Selection for growth is associated in gilthead sea bream (*Sparus aurata*) with diet flexibility, changes in growth patterns and higher intestine plasticity. *Aquaculture.* **2019**, 507, 349-360.
14. Janssen, K.; Chavanne, H.; Berentsen, P.; Komen, H. Impact of selective breeding on European aquaculture. *Aquaculture*. **2017**, 472, 8–16.
15. Dekkers, J.C.M. Blueprint: Breeding for resistance to infectious disease. *National Hog Farmer*. **2016**
16. Batargias, C.; Dermitzakis, E.; Magoulas, A.; Zouros, E. Characterization of six polymorphic microsatellite markers in gilthead seabream, *Sparus aurata* (Linnaeus 1758). *Mol Ecol Notes*. **1999**, *8*, 897–899.
17. Launey, S.; Krieg, F.; Haffray, P.; Bruant, J.S.; Vanniers, A.; Guyomard, R. Twelve new microsatellite markers for gilthead sea bream (*Sparus aurata* L.): characterization, polymorphism and linkage. *Mol Ecol*. **2003**, *3*, 457-459
18. Brown, R.C.; Tsalavouta, M.; Terzoglou, V.; Magoulas, A.; McAndrew, B.J. Additional microsatellites for *Sparus auarta* and cross-species amplification within the *Sparidae* family. *Mol Ecol*. **2005**, *5*, 605-607.
19. Navarro, A.; Badilla, R.; Zamorano, M.J.; Pasamontes, V.; Hildebrandt, S.; Sánchez, J.J.; Afonso, J.M. Development of two new microsatellite multiplex PCRs for three sparid species: Gilthead sea bream (*Sparus auratus* L.), red porgy (*Pagrus pagrus* L.) and red banded sea bream (*P. auriga*, Valenciennes, 1843) and their application to paternity studies. *Aquaculture*. **2008**, *285*, 30-37.
20. Porta, J.; Maria, J.P.; Bejar, J.; Carmen, M.A. Development of a microsatellite multiplex genotyping tool for the Gilthead sea bream (*Sparus aurata*): applicability in population genetics and pedigree analysis. *Aquaculture*. **2010**, *41*, 1514-1522.
21. Borrell, Y.J.; Gallego, V.; García-Fernández, C.; Mazzeo, I.; Pérez, L.; Asturiano, J.F.; Carleos, C.E.; Vázquez, E.; Sánchez, J.A.; Blanco, G. Assessment of parental contributions to fast- and slow-

growing progenies in the sea bream *Sparus aurata* L. using a new multiplex PCR. *Aquaculture*. **2011**, *314*, 58-65.
22. Magnadottir B. Innate immunity of fish (overview). *Fish Shellfish Immunol*. **2006**, 51, 20-137.
23. Whyte, S.K. The innate immune response of finfish–a review of current knowledge. *Fish Shellfish Immunol*. **2007**, 23, 1127-1151.
24. Navarro, A.; Oliva, V.; Zamorano, M.J.; Ginés, R.; Izquierdo, M.S.; Astorga, N.; Afonso, J.M. Evaluation of PIT system as a method to tag fingerlings of gilthead seabream (*Sparus Auratus* L.). Effects on growth, mortality and tag loss. *Aquaculture*. **2006**, *257*, 309–315.
25. Quade, M.J.; Roth, J.A. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Vet. Immunol. Immunopathol.* **1997**, 58, 3-4.
26. Oriol Sunyer, J.; Tort, L. Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are effected by the alternative complement pathway. *Vet. Immunol. Immunopathol*. **1995**, 45, 3-4.
27. Palenzuela, O.; Sitjà-Bobadilla, A.; Álvarez-Pellitero, P. Isolation and partial characterization of serum immunoglobulins from sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). *Fish Shellfish Immunol*. **1996**, 6, 81–94.
28. Lee-Montero, I; Navarro, A.; Borrell, Y.; García-Celdrán, M.; Martín, N.; Negrín-Báez, D.; Blanco, G.; Armero, E.; Berbel, C.; Zamorano, M.J.; Sánchez, J.J.; Estévez, A.; Ramis, G.; Manchado, M.; Afonso, J.M. Development of the first standardised panel of two new microsatellite multiplex PCRs for gilthead seabream (*Sparus aurata* L.). *Anim Genet*. **2013**, *44*, 533-546.
29. Peakall, R.; Smouse P.E. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*. **2012**, 28, 2537-2539.
30. Vandeputte, M.; Mauger, S.; Dupont-Nivet, M. An evaluation of allowing for mismatches as a way to manage genotyping errors in parentage assignment by exclusion. *Mol. Ecol. Notes*. **2006**, *6*, 265–267.
31. IBM Corp. Released. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp. **2017**.
32. Misztal, I; Tsuruta, S.; Lourenco, D.; Aguilar, I.; Legarra, A.; Vitezica, Z. Manual for BLUPF90 family of programs. University of Georgia, Athens, USA. **2015**.
33. R Development Core Team R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. **2016**.
34. García-Celdrán, M.; Ramis, G.; Manchado, M.; Estévez, A.; Afonso, J.M.; María-Dolores, E.; Peñalver, J.; Armero, E. Estimates of heritabilities and genetic correlations of growth and external skeletal deformities at different ages in a eared gilthead sea bream (*Sparus aurata* L.) population sourced from three broodstocks along the Spanish coasts. *Aquaculture*. **2015**, *445*, 33-41.
35. Carballo, C.; Shin, H.S.; Berbel, C.; Zamorano, M.J.; Borrego, J.J.; Armero, E.; Afonso, J.M.; Manchado, M. Heritability Estimates and Genetic Correlation for Growth Traits and LCDV Susceptibility in Gilthead Sea Bream (Sparus aurata). *Fishes.* **2020**, *5*, 2.
36. Antonello, J.; Massault, C.; Franch, R.; Haley, C.; Pellizzari, C.; Bovo, J.; Patarnello, T.; Koning, D.; Bargelloni, L. Estimates of heritability and genetic correlation for body length and resistance to fish pasteurellosis in the gilthead sea bream (*Sparus aurata* L.). *Aquaculture*. **2009**, *298*, 29-35.
37. Palaiokostas, C., Cariou, S., Bestin, A.; Bruant, J.S.; Haffray, P.; Morin, T.; Cabon, J.; Allal, F.; Vandeputte, M.; Houston, R.D. Genome-wide association and genomic prediction of resistance to viral nervous necrosis in European sea bass (*Dicentrarchus labrax*) using RAD sequencing. *Genet Sel Evol.* **2018**, *50*, 30.
38. Norris, A.; Foyle, L.; Ratcliff, J. Heritability of mortality in response to a natural pancreas disease (SPDV) challenge in Atlantic salmon, *Salmo salar* L., post-smolts on a West of Ireland sea site. *J Fish Dis*. **2008,** 31, 913-920.
39. Guy, D.; Bishop, S.C.; Woolliams, J.A.; Brotherstone, S. Genetic parameters for resistance to infectious pancreatic necrosis in pedigreed Atlantic salmon (*Salmo salar*) post-smolts using a Reduced Animal Model. *Aquaculture***. 2009,** 290, 229-235.
40. Ødegård, J.; Sommer, AI.; Kettunen Præbel, A. Heritability of resistance to viral nervous necrosis in Atlantic cod (*Gadus morhua* L.). *Aquaculture*. **2010a**, 300, 59-64.
41. Doan, Q.K.; Vandeputte, M.; Chatain, B.; Haffray, P.; Vergnet, A.; Breuil, G.; Allal, F. Genetic variation of resistance to Viral Nervous Necrosis and genetic correlations with production traits in wild populations of the European sea bass (*Dicentrarchus labrax*). *Aquaculture*. **2017,** 478, 1-8.

42. Bishop, S.C.; Woolliams, J.A. On the Genetic Interpretation of Disease Data. *PLOS ONE*. **2010**, 5,1.
43. Evenhuis, J.; Leeds, T.; Marancik, D.; LaPatra, S.; Wiens, G. Rainbow trout (*Oncorhynchus mykiss*) resistance to columnaris disease is heritable and favorably correlated with bacterial cold-water disease resistance. *J Anim Sci*. **2015,** 93, 1546-1554.
44. Guardiola, F.A.; Cuesta, A. & Esteban, M.Á. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol*. **2016**, 59, 323-330.
45. Lund, T.; Gjedrem, T.; Bentsen, H.B.; Eide, D.M.; Larsen, H.J.S.; Roed, K.H. Genetic variation in immune parameters and associations to survival in Atlantic salmon. *J Fish Biol*. **1995**, 46, 748-58.
46. Castanheira, M.F.; Conceição, L.; Millot, S.; Rey, S.; Bégout, M.L.; Damsgård, B.; Kristiansen, T.; Höglund, E.; Øverli, Ø.; Martins, C.I.M. Coping styles in farmed fish: consequences for aquaculture. *Rev Aquacult* **2015,** 9, 23-41.
47. Ferrari, S.; Millot, S.; Leguay, D.; Chatain, B.; Bégout, M. Consistency in European seabass coping styles: a life-history approach. *Appl Anim Behav Sci*. **2015**, 167, 74-88.
48. Dios, S.; Poisa-Beiro, L.; Figueras, A.; Novoa, B. Suppression subtraction hybridization (SSH) and macroarray techniques reveal differential gene expression profiles in brain of sea bream infected with nodavirus. *Mol. Immunol.* **2007**, 44, 2195-2204.
49. Fjalstad, K.T.; Moen, T.; Gomez-Raya, L. Prospects for the genetic technology in salmon breeding programmes. *Aquaculture Res*. **2003**, 34, 397-406.

CONCLUSIÓN

Las conclusiones obtenidas en nuestro estudio fueron:

En cuanto a **parámetros fenotípicos** la población F2_ATL presentó mayor peso corporal que la F0_MED, teniendo en cuenta que la primera ya había sido sometida a un programa de mejora a favor del peso y para eliminación de deformaciones. El peso tuvo una heredabilidad media. No se observó ningún efecto del proceso de selección en la heterocigosidad observada de F2_ATL, lo que quiere decir que no ha habido un incremento de la consanguineidad en F2_ATL.

Respecto a **resistencia** a Phd, no se obtuvieron diferencias entre ambas poblaciones para la tasa de mortalidad o los días hasta la muerte. La heredabilidad fue media para la resistencia y una baja para los días hasta la muerte. Por tanto, cabe esperar una buena respuesta cuando se seleccionan los reproductores a través de la descendencia para mejorar la resistencia a Phd.

Marcadores inmunológicos innatos, donde analizamos la actividad peroxidasa y bactericida y el nivel de IgM s, se observó mayor para actividad peroxidasa, y nivel de IgM para F2_ATL. La heredabilidad obtenida fue media para actividad peroxidasa y baja para actividad bactericida y nivel de IgM.

En las **correlaciones genéticas**, el peso estuvo positiva y altamente correlacionado con la resistencia a Phd. y los días hasta la muerte y, también se observó la tendencia a estar positivamente correlacionado con la actividad peroxidasa.

Se han obtenidos resultados de un programa de mejora genética (PROGENSA®), con los que se podría continuar mejorando a una tercera generación de doradas, que además de considerar el peso como criterio de selección, incluyese la resistencia a *Photobacterium damselae* subespecie *piscicida* a través de marcadores inmunológicos innatos, como la actividad peroxidasa, y que a su vez . Peces más grandes = peces más resistentes = peces con mayor actividad peroxidasa.

REFERENCIAS

1. Fish marine aquaculture in Spain. APROMAR. **2020**. (http://www.apromar.es)
2. Balebona, M.C.; Zorrilla, I.; Moriñgo, M.A.; Borrego, J.J. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Aquaculture*. **1998**, 166, 19-35.
3. Magariños, B.; Toranzo, A.E.; Romalde, J.L.; Phenotypic and pathobiological characteristics of *Pasteurella piscicida. Fish Dis.* **1996**, 6, 41-64.
4. Heuer, O.L.; Kruse, H.; Grave, K.; Collignon, P.; Karunasagar, I.; Angulo, F.J. Human Health Consequences of Use of Antimicrobial Agents in Aquaculture. *Food safety*. **2009**, 49, 1248–53.
5. Kumar, R.R.; Lee, J.T.; Cho, J.Y. Fate, Occurrence, and Toxicity of Veterinary Antibiotics in Environment. *J Korean Soc Appl Biol Chem*. **2012**, 55, 701−709.
6. Kovalakova, P.; Cizmas, L.; McDonald, T.J.; Marsalek, B.; Feng, M.; Sharma, V.K. Occurrence and toxicity of antibiotics in the aquatic environment. *Chemosphere*. **2020**, 251, 126351.
7. Flajnik, M.F., Du Pasquier L Evolution of the Immune System. 6 th ed. Paul WE, editor: Lippincott Williams & Wilkins. **2008**
8. Rubio-Godoy, M. Teleost fish immunology. Review. Revista Mexicana de Ciencias Pecuarias. **2010**, 1(1), 47-57.
9. Rauta, P.R., Nayak, B., Das, S. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunology letters*. 2012, 148(1), 23-33.
10. Tort, L., Balasch, J.C., MacKenzie, S. Fish health challenge after stress. Indicators of immunocompetence. *Contributions to Science*. **2004**, 2(4), 443-454.
11. Magnadóttir, B. Innate immunity of fish (overview). *Fish & shellfish immunology*. **2006**, 20(2), 137-151.
12. Magnadottir, B. Immunological control of fish diseases. *Marine biotechnology*. **2010**, 12(4), 361-379.
13. Valenzuela, C.A., Zuloaga, R., Poblete-Morales, M., Vera-Tobar, T., Mercado, L., Avendaño-Herrera, R., Valdés, J.A., Molina, A. Fish skeletal muscle tissue is an important focus of immune reactions during pathogen infection. *Developmental & Comparative Immunology*. **2017**, 73, 1-9.
14. Aoki, T., Takano, T., Santos, M.D., Kondo, H., Hirono, I. Molecular innate immunity in teleost fish: review and future perspectives. *In Fisheries for Global Welfare and Environment, Memorial Book of the 5th World Fisheries Congress*. **2008**, (pp. 263-276). Terrapub: Tokyo, Japan.
15. Whyte, S.K. The innate immune response of finfish–a review of current knowledge. *Fish & shellfish immunology*. **2007**, 23(6), 1127-1151.
16. Guardiola, F.A., Cuesta, A., Arizcun, M., Meseguer, J., Esteban, M.A. Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*). *Fish & shellfish immunology*. **2014**, 36(2), 545-551.
17. Cho, J.H., Park, I.Y, Kim, H.S., Lee, W.T., Kim, M.S., Kim, S.C. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *The FASEB Journal*. **2002**, 16(3), 429-431.
18. Dekkers, J.C.M. Blueprint: Breeding for resistance to infectious disease. *National Hog Farmer*. **2016**
19. Batargias, C.; Dermitzakis, E.; Magoulas, A.; Zouros, E. Characterization of six polymorphic microsatellite markers in gilthead seabream, *Sparus aurata* (Linnaeus 1758). *Mol Ecol Notes*. **1999**, 8, 897–899.
20. Launey, S.; Krieg, F.; Haffray, P.; Bruant, J.S.; Vanniers, A.; Guyomard, R. Twelve new microsatellite markers for gilthead sea bream (*Sparus aurata* L.): characterization, polymorphism and linkage. *Mol Ecol*. **2003**, 3, 457-459
21. Brown, R.C.; Tsalavouta, M.; Terzoglou, V.; Magoulas, A.; McAndrew, B.J. Additional microsatellites for Sparus auarta and cross-species amplification within the Sparidae family. *Mol Ecol*. **2005**, 5, 605-607.
22. Navarro, A.; Badilla, R.; Zamorano, M.J.; Pasamontes, V.; Hildebrandt, S.; Sánchez, J.J.; Afonso, J.M. Development of two new microsatellite multiplex PCRs for three sparid species: Gilthead sea bream (*Sparus auratus* L.), red porgy (*Pagrus pagrus* L.) and red banded sea bream (P. auriga, Valenciennes, 1843) and their application to paternity studies. *Aquaculture*. **2008**, 285, 30-37.

23. Porta, J.; Maria, J.P.; Bejar, J.; Carmen, M.A. Development of a microsatellite multiplex genotyping tool for the Gilthead sea bream (*Sparus aurata*): applicability in population genetics and pedigree analysis. *Aquaculture*. **2010**, 41, 1514-1522.
24. Borrell, Y.J.; Gallego, V.; García-Fernández, C.; Mazzeo, I.; Pérez, L.; Asturiano, J.F.; Carleos, C.E.; Vázquez, E.; Sánchez, J.A.; Blanco, G. Assessment of parental contributions to fast- and slow-growing progenies in the sea bream *Sparus aurata* L. using a new multiplex PCR. *Aquaculture*. **2011**, 314, 58-65.