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Aquaculture

Genetic parameters for *Photobacterium damselae* subsp. *piscicida* resistance, immunological markers and body weight in gilthead seabream (*Sparus aurata*)

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ARTICLE INFO

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

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., 2014). 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 and 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 (Kumar et al., 2012; Kovalakova et al., 2020).

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 (Massault et al., 2010; Palaiokostas et al., 2016; Janssen et al., 2017).

Previous studies in seabream demonstrated that *Phdp* resistance showed an oligogenic-polygenic architecture and highlighted some associated QTLs (Massault et al., 2010; Aslam et al., 2014; 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; Massault et al., 2010; Aslam et al., 2014; 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 bio-markers 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.

2. Materials and methods

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).

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 (8 L: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 (5000 L). Another larvae sample from F0_MED was placed in another tank (5000 L). 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 h and centrifuged at 10,000 $\times g$ for 10 min at 4 °C to obtain the serum that was frozen at −80 °C until further analysis, as previously described (Sunyer and Tort, 1995; Hernández and Tort, 2003; Cuesta et al., 2004). 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.

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 h. The exponentially growing culture was washed three times with sterile phosphate buffered saline (PBS) by centrifugation at 2700 $\times g$ for 15 min 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 8×10^5 cfu/mL.

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 8×10^5 cfu/mL, a sublethal dose of *Phdp* (8×10^4 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).

2.5. Recorded traits

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

2.5.2. 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.

2.5.3. 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 M* (IgM) as marker of adaptive humoral immune status in serum samples.

2.5.3.1. 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 (Multi-skanGo, 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.

2.5.3.2. 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 10^8 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.

2.5.3.3. Total levels of immunoglobulin M.
A direct ELISA was used to detect total IgM in gilthead seabream serum. For IgM detection, Maxi-iSorp 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 1:1000 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).

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.

2.7. Statistical analysis

2.7.1. For phenotypical analysis

Numerical data for each trait were tested for normality and homogeneity of variances using SPSS® (v.25.0) (IBM Corp, 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

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 $P < 0.05$.

2.7.2. For genetic analysis

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

$$Y = X\beta + Zu + e$$

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 markers*,

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:

$$P(\beta) \sim k$$
$$P(u|G) \sim (0, G \otimes A) \qquad (1)$$
$$P(e|R) \sim (0, R \otimes A)$$

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

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

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 Development Core Team (2020). 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.

3. Results

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 (Fig. 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.

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 (Fig. 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 (Fig. A1).

The *BW* effect on *Mortality rate* was significant, but this effect was not linear, the logarithmic transformation was needed to reveal that higher log*BW* 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 (Fig. 3).

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.

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

Table 1

Phenotypic results (least square means ± standard error) for body weight and *immunological markers* for juvenile gilthead seabream from two populations.
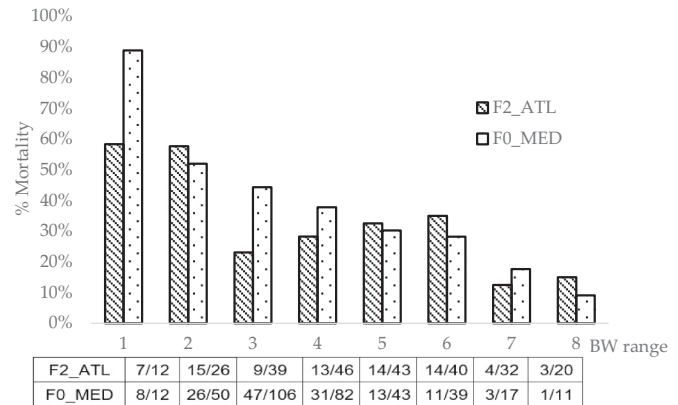
Broodstocks origin[1]	F2_ATL			F0_MED			Cov BW	
	n	LSM	S.E.	n	LSM	S.E.	b	S.E.
BW (g)	325	16.9[a]	0.28	368	13.2[b]	0.27	–	–
Days to death	93	2.13	0.12	141	2.23	0.10	0.019	0.019
Peroxidase activity (U.I./mL)	285	38.2[a]	1.65	359	15.7[b]	1.46	−0.308*	0.194
Bactericidal activity (%)	143	17.8	0.98	294	18.8	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

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.
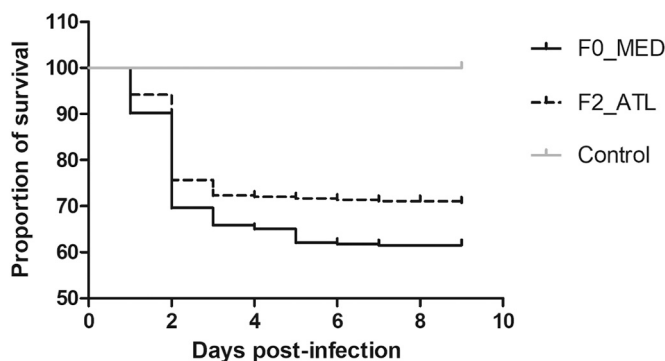
[1] Broodstocks origin: F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population.
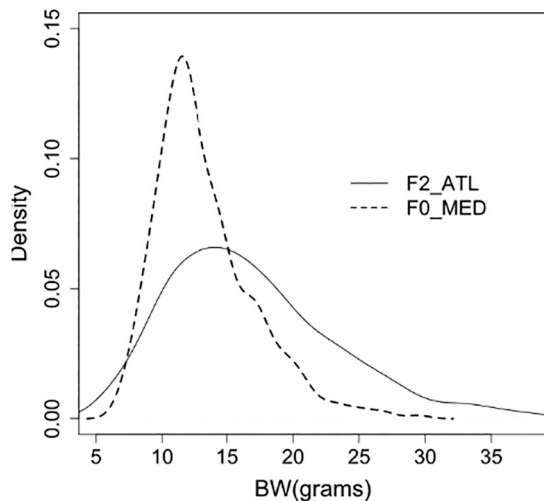
Fig. 1. *Body weight* (*BW*) at 250 dph distribution for both populations. F2_ATL = F2 Atlantic Ocean population (n = 325) and F0_MED = F0 Mediterranean Sea population (n = 368).

Fig. 2. Kaplan-Meier survival curves showing the proportion of F0_MED and F2_ATL survivors after intraperitoneal injection with 8x10⁴ cfu/fish and a non-infected (control) group. F2_ATL = F2 Atlantic Ocean population and F0_MED = F0 Mediterranean Sea population.

Fig. 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.

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.
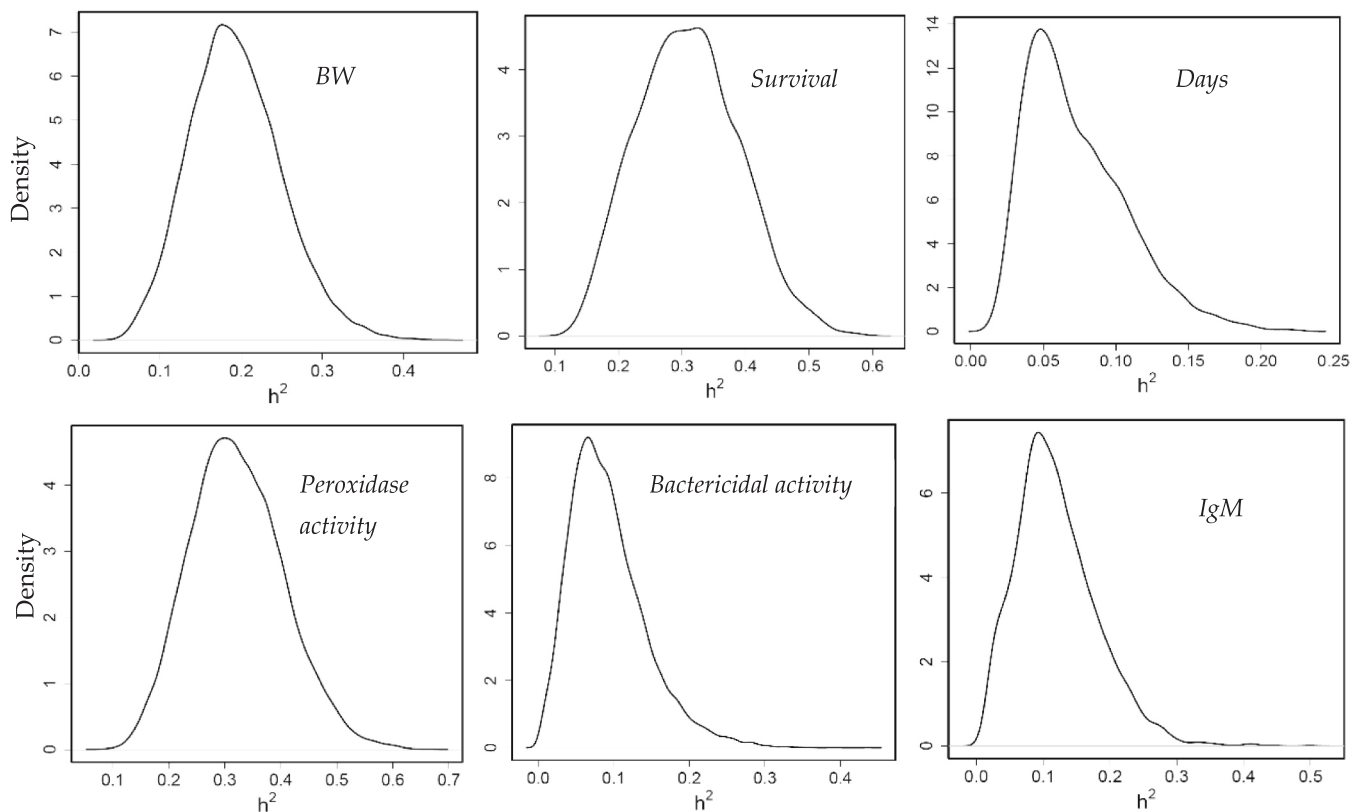
3.3. Genetic parameters

3.3.1. 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* (Fig. 4).

3.3.2. 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

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♀.

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.

Fig. 4. Posterior marginal distribution of heritabilities of body weight (*BW*), *Photobacterium damselae* survival, 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.

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) (Fig. 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.

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 (García-Celdrán et al., 2015; Carballo et al., 2020). 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 8×10^4 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 1×10^5 cfu of *Phdp*/100 L, 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 3×10^6 cfu of *Phdp*/100 L. Aslam et al. (2014) with fish 3–4 g in weight and 120 dph infected by intramuscular inoculation with
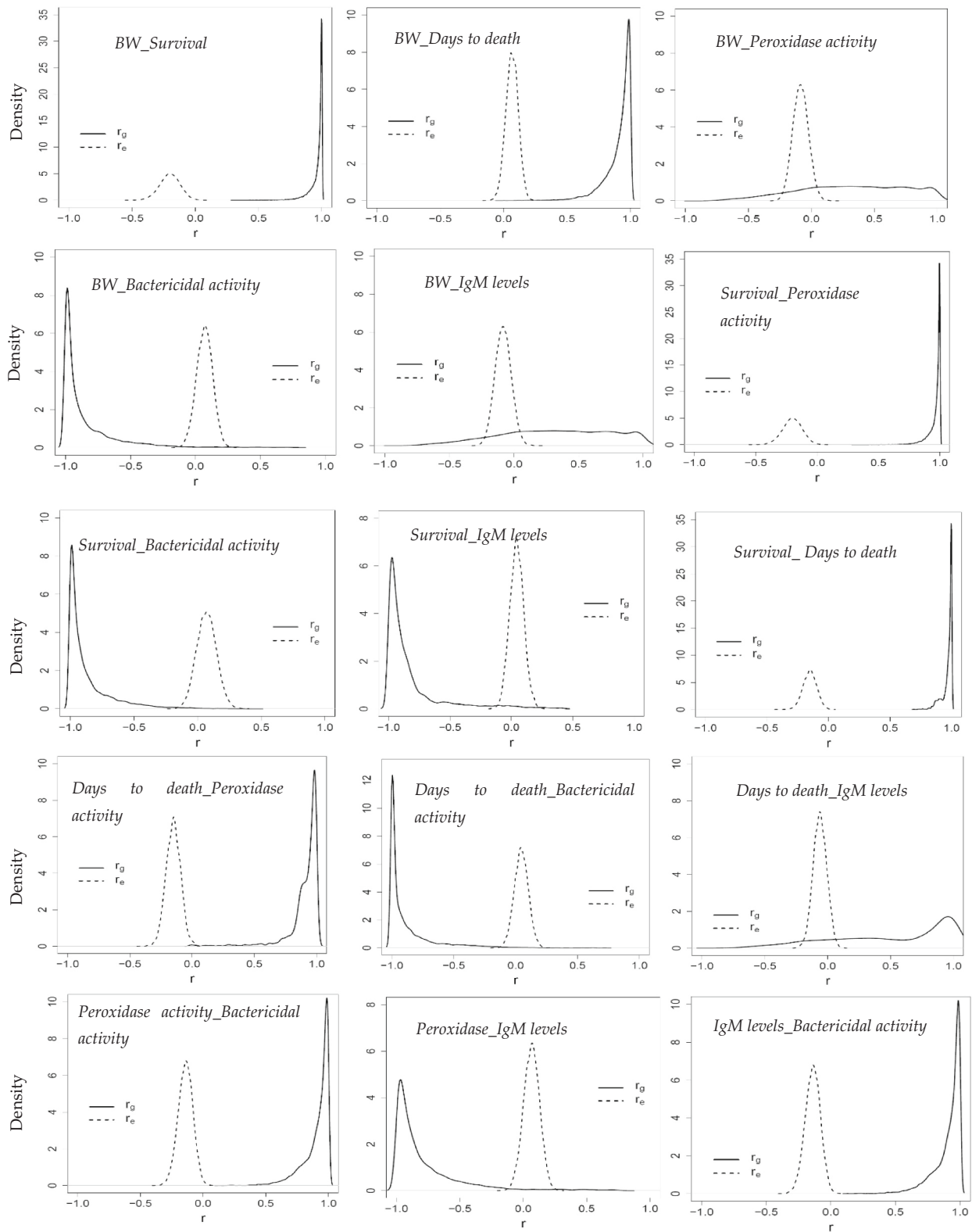
Fig. 5. Posterior marginal distribution of genetic correlations of *body weight* (*BW*), *Photobacterium damselae* resistance, *days to death and immunological markers* (*peroxidase activity, bactericidal activity and immunoglobulin M (IgM) levels*) gilthead seabream. rg = genetic correlation; re = residual correlation. All measure with 609 data, except to *bactericidal activity* with 457 data and *IgM* with 566 data.

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.

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.

Author contributions

Elena Chaves-Pozo and Marta Arizcun bred the animals and carried out the infection of the pathogen and collected data for further analysis; Rubén Pérez and Antonio Vallecillos did the genetic analysis in the laboratory; Antonio Vallecillos data interpretation and preparation of the manuscript; Antonio Vallecillos and Eva Armero performed statistical analysis. Eva Armero preparation of the manuscript and supervision; Juan Manuel Afonso project administration and supervision; Manuel Manchado, Jaume Pérez-Sánchez and Emilio María-Dolores Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736892.

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