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Article

Advanced Genomic Analysis of Growth and Disease Resistance in Gilthead Sea Bream (Sparus aurata): Elucidating Heritability and Genetic Correlations

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Abstract: The study investigates the genetic factors associated with lymphocystis disease virus (LCDV) susceptibility and growth in gilthead sea bream aquaculture, considering two fish batches (batch 1 and batch 2). All specimens exhibited LCDV lesions at the time of sampling. Batch 1 displayed higher lesion severity and affected surface area compared to batch 2. The caudal region was least affected in both batches. Batch 1 also had higher LCDV DNA copies in the liver, positively correlated with severity index. Heritabilities for weight, length, and viral DNA copies were determined, showing low heritability for viral DNA copies in both batches. Selective breeding for LCDV susceptibility and growth is feasible in sea bream juveniles, with genetic correlations indicating the possibility, though estimates vary with age. This information is valuable for designing selective breeding programs in sea bream.

Keywords: Gilthead Sea Bream; Genetics; Growth Traits; Disease Susceptibility

1. Introduction

Worldwide, the aquaculture business suffers significant economic losses due to the extremely contagious viral virus known as lymphocystis disease (LCD) [1]. The lymphocystis disease virus (LCDV), which is a member of the Iridoviridae family and the genus Lymphocystivirus, is the culprit. Asymptomatic carriers, who are infected fish, can go for many times without showing any symptoms of Iridoviridae until their host immunocompetence drops for various reasons such temperature changes, grading procedures, transportation, or dense tank populations. Lymphocysts, the characteristic lesions, are formed when the virus multiplies in host cells. They are white masses that are irregularly shaped and dispersed randomly across the integumentary surfaces. In cases of severe infections, these masses can cluster into nodular forms [2]. It takes at least three weeks following an LCDV injection before clinical indications can be observed, according to experimental trials [3,4]. There is a high rate of morbidity but a low risk of fatality unless additional infections arise within the roughly 20 days after the lesions emerge, which is the time it takes for the hypertrophy dermal cells to mature and rupture [5]. Due to the significant delay in fish growth, reduced feed conversion rates, and the inability to commercialise fish due to external lesions and consumer rejection, LCDV infection has a significant economic impact in hatcheries, despite being a self-limiting benign process [3].

The lack of effective commercial vaccinations has prompted the establishment of multiple strategies for the control of LCDV outbreaks in hatcheries. The most common strategy is keeping an eye out for asymptomatic carriers and eliminating them using very sensitive diagnostic tools [6–8]. While this approach has been effective in halting transmission in one direction, it necessitates constant monitoring of broodstocks and does little to stop transmission in another direction. Alternatively, immunostimulants delivered by food can boost the immune response and prevent the virus from replicating [9,10]. Planning stressful operations in the hatcheries and implementing preventive measures is where this technique really shines. Thirdly, resistant lineage genetic selective breeding is an option to consider. A significant locus linked to resistance against LCDV genotype II was found in Paralichthys olivaceous [11], which was utilised to create marker-assisted breeding programmes and LCDV-resistant broodstocks [12]. This second approach necessitates the assessment of genetic

variation in populations peculiar to each species, but it has emerged as one of the most long-term solutions to the problem of LCDV outbreaks in aquaculture.

Among the many fish species farmed in the Mediterranean region, gilthead sea bream (Sparus aurata) ranks high. Similar to other Mediterranean species as Senegalese sole, this one has genotype VII infection [13,14]. Annually, LCDV outbreaks are common and, on rare occasions, cause a lot of deaths [15]. Improving development, morphology, and carcass quality are the main goals of most active genetic programmes for this species [16,17]. Only the bacterial pathogen Photobacterium damselae subsp. piscicida has had genetic estimations for disease resistance published [18,19]. Nevertheless, the exact genetic variant that determines sea bream's vulnerability to LCDV is still a mystery. We set out to quantify heritabilities and other genetic characteristics in our research.

2. Results

2.1. Biometric Data

The vulnerability of two experimental fish batches (batch 1 and batch 2) to LCDV was assessed during a disease outbreak in a commercial hatchery. The fish batches were created by collecting eggs over a period of four consecutive days (using the 4DL model) from three sea bream broodstocks. These batches were consistently handled as a single unit, following the same culture conditions, from the larval stages until the occurrence of a disease outbreak and subsequent sampling. During the disease outbreak, the average weight for batch 1 was 1.3 ± 0.5 g at around 80 days after hatching, while for batch 2 it was 3.8 ± 1.1 g at around 140 days after hatching ($p < 0.05$; Figure 1. The average length for batch 1 was 3.8 ± 0.4 cm, but for batch 2 it was 5.3 ± 0.4 cm ($p < 0.05$; Figure 1).

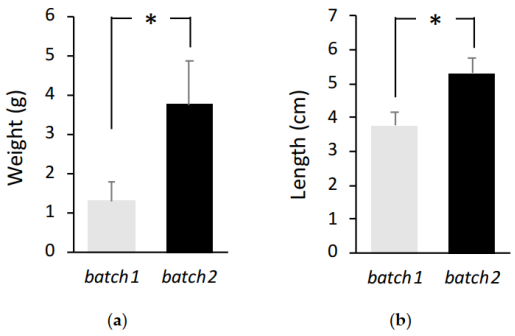

Figure 1. Displays the weight (a) and length (b) measurements for batch 1 and batch 2. The mean value plus or minus the standard deviation is provided, with a total sample size of 498 for each batch. The asterisk indicates notable distinctions among batches.

2.2. Disease Phenotyping

All animals that were collected exhibited external skin lesions that were consistent with the LCDV infection, resulting in a morbidity rate of 100%. In order to determine the proportion of the surface area affected by lesions and their severity, photographs of each animal were taken and subsequently subjected to image processing for three specific locations (R1-R3) (see to Section 4.2 and Figure S1). The phenotype data for surface and intensity attributes are illustrated in Figure 2. The Wilcoxon test revealed a statistically significant difference between the surface areas covered in batch 1 and batch 2 ($p < 0.05$). The average surface area covered was 2.55 for batch 1 and 2.49 for batch 2. The examination of the body region indicated that regions 1 and 2 exhibited a greater degree of impact on their surfaces (2.73 ± 0.47 and 2.66 ± 0.55) compared to region 3 (2.27 ± 0.77) ($p < 0.05$) in batch 1 (Figure 2). A significant gradient was seen in batch 2, with region 1 (2.86 ± 0.35) showing a higher value compared to region 3 (1.92 ± 0.83) ($p < 0.05$). The intensity of lesions in batch 1 was considerably higher than in batch 2 (average 1.9 ± 0.7 vs. 1.5 ± 0.6) (Figure 2), with a p-value of less than 0.05. There were no variations in the intensity levels across different regions.

The observed values for batch 1 ranged from 1.79 to 1.93, while for batch 2 they ranged from 1.4 to 1.6.

In order to more accurately describe the genetic variation and the relationships between productive qualities, the severity index (SI) was employed to categorise the animals into four distinct groups: Weak, moderate, severe, or extremely severe (Figure 3). The mean SI was 5.0 ± 2.6 and 3.9 ± 2.2 for batch 1 and batch 2, respectively. Batch 1 had a prevalence of severe or very severe infection in 60% of the fish, however in batch 2, this prevalence decreased to 40% (Figure 3).

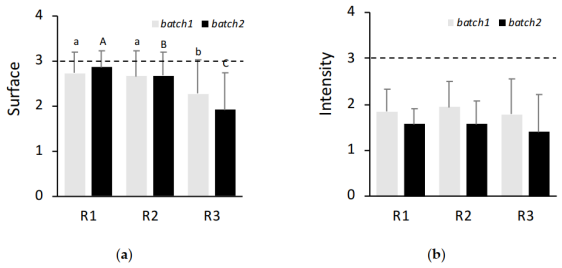

Figure 2. The surface coverage rates (a) and lesion intensity (b) for regions (R) 1, 2, and 3 were measured in both batch 1 and batch 2. The dashed line represents the highest possible rate value. The letters denote notable variations between regions for either batch 1 (in lowercase) or batch 2 (in uppercase). The mean value together with its corresponding standard deviation is provided.

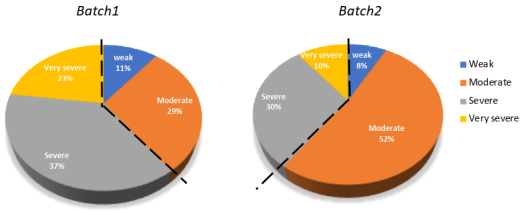

Figure 3. Classification of severity index (SI) in batch 1 and batch 2. The percentages for the four categories are stated. The dashed line represents the binary classification of fish susceptibility, distinguishing between low (weak and moderate) and high (severe and extremely severe) susceptibility.

2.3. Quantification of Viral DNA Copies

The liver of fish from batch 1 and batch 2 was analysed to determine the quantity of viral DNA copies (Figure 4). In batch 1, the average number of LCDV copies was 9.2 × 107 ± 7.2 × 108 μg total DNA-1, while in batch 2 it was 6.3 × 105 ± 5.9 × 106 μg total DNA-1. Significantly, a strong and positive association was found between the average quantity of viral DNA particles in the liver and the SI categories (r^2 = 0.90 – 0.94), as shown in Figure 4.

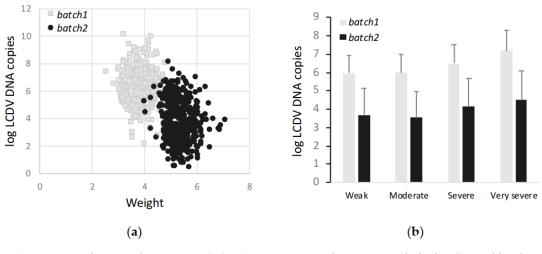

Figure 4. The number of Lymphocystis disease virus (LCDV) DNA copies is represented on a logarithmic scale for batch 1 and batch 2. (a) The amount of DNA copies increases in relation to weight; (b) The amount of DNA copies increases in relation to four SI categories.

2.4. Parental Contribution and Genetic Estimates

Out of the offspring in batch 1, 95.9% were attributed to a specific breeder, while 86% were linked to a distinct set of parents. The percentages in batch 2 were 93.4% and 84.0%. The total number of families was 150 for batch 1 and 128 for batch 2. Among these, 87% of the offspring were assigned to the F2 broodstock, 9.7% were assigned to the F1 + F2 broodstock, and 3.3% were assigned to the non-selected control broodstock. Out of the 82 breeders, 54 of them produced offspring, however there was a significant bias in the percentage of contribution (Figure 5). The reconstructed breeder sex ratio for producing offspring was 1 male to 2.8 females for batch 1 and 1 male to 1.6 females for batch 2. Males exhibited a much greater bias ($p < 0.05$) in the amount of offspring contributed per breeder compared to females. The maximum percentages for males were 31% and 51% for batch 1 and batch 2, respectively. In contrast, females only contributed 9%

and 16% for batch 1 and batch 2, respectively. The examination of SI and the quantity of LCDV DNA copies per family revealed a significant range of values, with batch 2 displaying even more pronounced variation (Figure 5). The Standard Index (SI) varied from 1.7 to 9 in both batches, while the logarithmic number of viral DNA copies ranged from 4.6 to 7.9 in batch 1 and from 1.8 to 6.2 in batch 2.

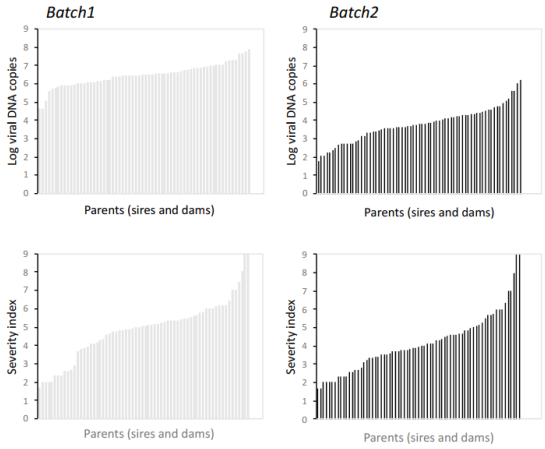

Figure 5. The logarithmic scale displays the variation in LCDV DNA copies, as well as the severity index of sire and dam families for batch 1 (left panel) and batch 2 (right panel).

The table displays the heritability and genetic association estimates for weight, length, LCDV DNA copies, and SI in binary scale (SI). The heritabilities for weight and length in batch 1 were 0.18 and 0.14, respectively. In batch 2, these values reduced to 0.06 and 0.05. The heritability of the amount of viral DNA copies was found to be low in both batches, with values of 0.04 and 0.08 for batch 1 and batch 2, respectively.

In order to assess the genetic vulnerability to LCDV infection using the threshold model, animals were categorised into two groups based on their SI: Weak/moderate and severe/very severe. The heritability for the binary SI was 0.20 ± 0.10 in batch 1 and 0.14 ± 0.08 in batch 2, as measured on the observed scale. Upon conversion to the liability scale, the heritabilities exhibited an increase to 0.32 and 0.21, respectively. A Bayesian linear mixed model was utilised to estimate the heritability by employing the binary-coded LCDV susceptibility data. The Bayesian narrow-sense heritability estimate for batch 1 was 0.33, and for batch 2 it was 0.24. The 95% Bayesian credibility range for the additive genetic component was 0.12-0.67 for batch 1 and 0.01-0.53 for batch 2 (Figure 6).

Table 1 presents the genetic and phenotypic connections among weight, length, LCDV DNA copies, and SI. The genetic connections between growth traits (weight and length) and between disease traits (number of LCDV DNA copies and SI) were found to be significantly high and positive in both batches. Nevertheless, after examining the development and illness characteristics, the genetic associations were found to be positive and moderately to highly significant in batch 1, but negative in batch 2. It is worth mentioning that the model achieved convergence for length and SI, but did not generate any error values. The phenotypic correlations between weight and length were strongly positive, while the correlations between LCDV DNA particles and SI were somewhat favourable. The phenotypic correlations between biometric and susceptibility features were nearly nonexistent in batch 1, whereas in batch 2, they were both low and negative.

3. Discussion

One long-term plan to encourage aquaculture growth is to use selective breeding to make fish more resistant to diseases. Disease resistance characteristics in fish have moderate to high heritabilities, according to previous studies. The incorporation of these characteristics into breeding programmes can thereby decrease fish mortality and increase fish health [20]. The majority of sea bream genetic improvement efforts have focused on morphology, growth, and carcass quality [21,22]. Only genes related to resistance to the pathogenic bacterium P. damselae subsp. piscicida have been studied thus far, and their values range from 0.12 to 0.28 [19,20]. According to previous studies on P. olivaceous and LCDV, marker-assisted selection programmes were successful in controlling outbreaks of LCDV-C (genotype II) [12]. Due in large part to the difficulties in conducting experiments, there is presently no publicly available evidence regarding the genetic variation for resistance to LCDV, despite its frequent observation in sea bream hatcheries.

Table 1. The heritabilities are indicated in bold, whereas the phenotypic correlations are shown below the diagonal and the genetic correlations are shown above the diagonal. The data presented includes the mean values and standard deviations (SD) for body weight, fork length, LCDV DNA copies, and SI (binary scale)

Batch 1	Weight	Length	DNA Copies	SI
Weight	0.28 ± 0.10	0.88 ± 0.03	0.60 ± 0.68	0.77 ± 0.30
Length	0.72 ± 0.01	0.09 ± 0.09	0.44 ± 0.65	0.54 ± 0.39
DNA copies	-0.02 ± 0.05	-0.02 ± 0.05	0.06 ± 0.05	0.99 ± 0.35
SI	0.20 ± 0.05	0.01 ± 0.05	0.42 ± 0.05	0.30 ± 0.10
-	-	-	-	0.32 ± 0.16
Batch 2	Weight	Length	DNA Copies	SI
Weight	0.08 ± 0.07	0.99 ± 0.12	-0.96 ± 0.64	-0.79 ± 0.65
Length	0.99 ± 0.01	0.08 ± 0.08	-0.99 ± 0.72	-0.80 *
DNA copies	-0.14 ± 0.05	-0.21 ± 0.05	0.09 ± 0.08	0.99 ± 0.43
SI	-0.27 ± 0.05	-0.29 ± 0.05	0.30 ± 0.05	0.14 ± 0.08
-	-	-	-	0.21 ± 0.09

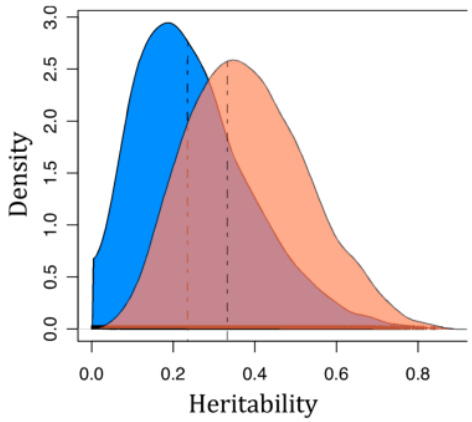

Figure 6. Posterior distributions of the heritability estimates for LCDV susceptibility in batch 1 (red) and batch 2 (blue) generated from Bayesian MCMCglmm models. Dashed lines correspond to distribution modes.

Both the fish's immune system and its immediate environmental conditions significantly impact the disease's potential to spread. A commercial hatchery with a history of LCDV outbreaks generated two batches of fish from mass spawning of three different broodstocks. All other aspects of the production process, including the grading operations, were maintained constant for the two batches of fish. Fish samples were collected for phenotyping and genotyping using a cross-sectional technique at the exact moment when distinctive LCDV skin lesions were visible. This was done with the assumption that LCDV infections had occurred at least three weeks earlier [3].

Considering the minimal mortality rates linked to LCDV outbreaks, it is recommended to measure clinical signs, such as the area and severity of lesions, in order to determine disease susceptibility [2,3,5,6]. The presence of distinctive LCDV lesions in all animals did not result in any deaths during the sampling period of our study (Figure 6). An image analysis was performed to assess the severity of the infection, and it revealed a great deal of variation in the location and brightness of the lesions across the body. In order to establish a correlation with the intensity of clinical symptoms, the number of LCDV DNA copies was also computed. The presence of viral MCP protein [8] was confirmed by the qPCR analysis in all animals, which was expected given the lesions. Nevertheless, the virus loads varied considerably. Moreover, this index is a dependable measure of viral replication because of the high relationship (>90%) seen between the quantity of LCDV DNA copies and the four SI categories. Though it is not the principal organ for LCDV viral replication, this virus can replicate on the liver before progressing to the integumentary system [3,14,24]. To avoid cross-contamination among skin lesions in fish populations with high infection rates, the choice of this organ is particularly critical for precisely counting DNA copies.

An interesting feature revealed by the reconstruction of the family tree is the high degree of variation in the contributions made by the progeny.

It has been shown in the past that sea bream broodstocks managed by photoperiod display mass-spawning behaviour [20,25]. Although 59% of breeders in this study produced offspring, more female breeders followed the broodstock structure that was set up at the beginning of the breeding period. Despite having the same number of breeders in both groups, the most striking finding was the clear preference for offspring contribution in the F2 group compared to the control broodstocks. This theory is not fully supported by the quadratic relationship between parental weights and offspring output [25], even though the weight difference between breeders (average 1875 ± 240 vs. 731 ± 144 for F2 and control, respectively) could potentially explain the variations in egg production. Selective breeding for increased F2 production might have improved relative fecundity in a roundabout way. Nevertheless, additional testing is necessary to validate this concept.

Both batches' extremely low heritability estimates for the hepatic number of LCDV DNA copies (0.04 and 0.08) suggest that genetic factors had no impact on this feature. Nevertheless, a value greater than 0.99 indicated a robust genetic correlation with SI. On the other hand, batch 2 had a heritability of 0.22-0.24, and batch 1 had a heredity of 0.32-0.33. These numbers are based on the liability scale. This indicates that this index has the potential to be an important trait for determining LCDV susceptibility in families. In the European sea bass (0.26 ± 0.11) or against P., the heritabilities showed modest values that were similar to those reported for nodavirus resistance on the liability scale.

According to research [20,21], sea bream had a prevalence of the bacterium Damselae subspecies piscicida ranging from 0.12 to 0.28 percent. Because of this, it's probable that selective breeding played a role. We have proven that LCDV susceptibility is feasible.

Heritabilities for furcal length in juvenile sea bream fish are moderate, according to previous research. The heritability is predicted to be 0.31 ± 0.07 for 6.6 cm long fish that are 130 days old after hatching, according to reference [22]. Likewise, the heritability is predicted to be 0.38 ± 0.08 for fish that are around 2.3 cm long and that are 110 days old after hatching [20]. The weight heritability was found to be 0.28 ± 0.07 at 130 days post-hatch, according to Navarro, Zamorano, Hildebrandt, Gines, Aguilera, and Afonso [23]. Our study's fish length and weight values are very similar to those of Navarro et al. (2009). Nevertheless, in batch 1, our heritability estimates for weight were 0.18 and for length, they were 0.14; in batch 2, they were nearly nil. One possible explanation for the drop in genetic estimates is that LCDV redistributes energy away from somatic growth and towards viral replication and the overgrowth of fibroblasts in the integumentary tissues, hence reducing genetic estimations. Furthermore, differences in heritability between groups of fish of different ages, weights, and SI suggest that bigger fish can divert their energy towards fighting off LCDV infections, thus masking the effects of genetics on growth.

According to the theory, the SI in both groups is correlated with their weight and length, two measures of growth. In particular, batch 1 has a positive and high correlation, but batch 2 has a negative correlation. A major shortcoming of this study is that it does not include any longitudinal data that spans the whole duration of the infection. The results of the study, which compared two age groups with varied genetic links, provide solid evidence that the likelihood of infection increases with fish age. A person's ability to fend off viral infections is inversely proportional to their weight [24,25]. Fork length was found to be positively correlated with resistance to P. damselae subsp. piscicida in sea bream, according to the authors. Nonetheless, they failed to ascertain whether the correlation was impacted solely by weight or survival duration [20]. Our research showed that there was a lot of weight fluctuation across batches, and that changes in genetic association were more likely associated with growth and possible improvements in immune response than with size alone. Because of this, fish are able to fight off this innocuous disease. If this theory is correct, then selecting for animals with higher SI and longer statures could be easier using a hybrid selection index. This method would limit viral replication and phenotypic abnormalities while preserving the ability to expand.

4. Materials and Methods

4.1. Animals

Three broodstocks, located at centre IFAPA El Toruno in El Puerto de Santa Maria, Spain, and belonging to the Spanish genetic breeding programme PROGENSA, were utilised for family production. (a) The group of animals that were not chosen or used as a control group consisted of 52 breeders who were 4 years old. (b) The group of broodstock for the first and second generations that were selected for growth included 30 breeders. The females were 7 years old and the males were 4 years old. (c) The group of broodstock for the second generation that were selected for growth consisted of 57 breeders who were 4 years old. The tanks had an estimated female-to-male ratio of 2:1. The three broodstocks were subjected to a regulated light-dark cycle (8 hours of light and 16 hours of darkness) in order to coordinate the process of

maturation and release of eggs. This cycle was began in early December 2016. Throughout this time frame, animals were provided with unrestricted access to Vitalis Cal (Skretting) feed, and the daily egg production was closely observed. Once the overall egg production reached a consistent level, two separate egg batches were created: one at the conclusion of February and another at the beginning of April 2017. For both scenarios, eggs from the three broodstocks were gathered and combined over a period of four consecutive days. This was done to optimise family production based on the 4DL model (Elalfy et al., unpublished). The incubation process took place in cylindrical conical tanks with a capacity of 1000 litres, with a larval density ranging from 500 to 1000 larvae per litre. The water conditions were as stated: The temperature is 19.0 ^{o}C, the salinity is 34%0, and the dissolved oxygen concentration is 6.4 mg L-1. PCR analysis was conducted on all spawns to verify the lack of LCDV infections, as previously documented [8]. Due to the LCD's strong reliance on environmental conditions, it is challenging to replicate it in a laboratory setting. Therefore, we sent healthy non-hatched eggs and hatched larvae to an industrial hatchery in Huelva, Spain, where LCDV outbreaks have previously occurred. The larval batches were raised in 5 m3 tanks at an initial density of 100 larvae L-1. The rearing techniques employed in the hatchery were conducted on a commercial scale. These two batches were consistently treated as experimental units and were not sorted until the sampling process.

The LCDV epidemic took place in June 2017, while the temperature was 26^{o}C and the salinity was 36 ppt. During the sample process, the animals exhibited obvious lesions that were consistent with LCDV infections. Since our animals were raised in controlled industrial circumstances and all tanks were managed uniformly, there were no tanks with undesirable outcomes. The presence of LCDV infection was verified using quantitative polymerase chain reaction (qPCR), and the extent of lesion intensities was assessed by image analysis. Each batch was subjected to random sampling, resulting in a total of 500 animals. These animals were then sacrificed in slurring ice to preserve any exterior wounds. Subsequently, they were sent to the IFAPA "El Toruno" centre for phenotyping and samplings. The two groups were designated as batch 1 (eggs collected in April; approximately 80 days after hatching) with a significant quantity of typical lymphocysts, and batch 2 (eggs collected in February; approximately 140 days after hatching) with less noticeable lesions, but all displaying the classic signs of LCDV infection.

4.2. Data Collection and Image Analysis

Stringent biosecurity procedures were implemented to prevent the spread of viruses among the animals at the facilities. Each animal was taken separately using a Canon EOs1300D camera, following the stated protocol in PROGENSA® [19]. Subsequently, the animals were weighed and individually placed in a freezer at a temperature of -20^{o}C for future tissue sample. The Bioethics and Animal Welfare Committee of IFAPA granted authorization for all procedures.

The Fiji 2.0.0-rc-69/1.52i software was utilised for image analysis. The furcal length of all fish specimens was measured, and subsequently, the extent of surface covered by lesions and their intensity were assessed by dividing the animals into three corporal sections (Figure 6). Region 1 (R1) extends from the back section of the operculum to the tip of the pectoral fin. Region 2 (R2) extends from the tip of the pectoral fin to the base of the caudal fin. Region 3 (R3) refers to the caudal fin. The ranking of each region was determined based on the extent of lesion coverage: a score of 1 was assigned if the lesions covered less than 30% of the surface, a score of 2 if the coverage was between 30% and 70%, and a score of 3 if the coverage exceeded 70% (Table 2). The skull was excluded from consideration due to the challenges associated with accurately visualising the lesions. In addition, lesion intensities were determined for each region as follows: a score of 1 if the lesions were not conspicuous, a score of 2 if they were mildly noticeable, and a score of 3 if lymphocysts were clearly visible (Table 2). Each animal had separate assessments by two researchers to evaluate their phenotypic features, including surface and intensity. The evaluations from both researchers were then combined to provide a consensus rating for each animal. Furthermore, the severity index was determined by calculating the surface area (S) affected by lesions and their level of intensity (I) using the following formula: The value of SI is calculated by multiplying the mean values of S and I from the three locations. The index fluctuates within a range of 1 to 9 and was employed to establish four distinct groups for genetic analysis: Weak, moderate, severe, or very severe (Table 2).

4.3. Quantification of Viral DNA

While the skin has the largest viral DNA copy count, LCDV also reproduces in the liver [22,23]. The chosen organ for quantifying the viral DNA copies in each specimen is the target organ. This is because it offers sufficient biomass for nucleic acid isolation, hence reducing the danger of cross-contamination across individuals. The livers were dissected using sterile techniques, sorted into individual eppendorf tubes, and stored at a temperature of -20^{o}C until

Table 2. Rates (R) for LCDV lesions according to surface covered and intensity. Categories for severity index are also shown

	Surface		Intensity	Severity Index (SI)
R	%	R	Type	R Category
1	<20	1	White layer but not prominent	1-3 weak
2	20-60	2	Slightly prominent	3-6 moderate
3	>60	3	Lymphocysts	6-9 severe
-	-	-	-	8-9 very severe

further processing. The materials underwent cleaning using ethanol and distilled water after each animal. For batch 1, the entire DNA was extracted using the Isolate II Genomic DNA Kit from Bioline. In batch 2, the DNA samples were purified using phenol/chloroform/isoamyl alcohol from Sigma, following the directions provided by the manufacturer. The DNA samples underwent treatment with RNase A (Bioline) and were measured using spectrophotometry with the Nanodrop ND-8000. The measurement of viral DNA copies was performed using the procedure outlined by Valverde, Cano, Labella, Borrego, and Castro [8], which relies on the detection of the main capsid protein (mcp). The assays were conducted using a CFX96TM Real-Time System (Bio-Rad) with a final volume of 10 μL. The assay mixture consisted of 200 ng of DNA, 300 nM of both forward and reverse primers, and 10 μL of iQ^{TM} SYBR® Green Supermix (Bio-Rad). The amplification protocol employed was as follows: The process begins with a 7-minute denaturation and enzyme activation at a temperature of 95^{o}C. This is followed by 40 cycles, each consisting of 30 seconds at 95^{o}C and 1 minute at 59^{o}C.

4.4. Determining genetic variations and assigning parentage

The DNA used for determining the parentage of the offspring was extracted from the liver, using the methods described in Section 2.3. Prior to the spawning season, breeders were selected for sampling. A little amount of blood (about 0.5 mL) was collected by puncturing the caudal vein using a syringe containing heparin. The blood samples were then treated with 100 mU of heparin and stored at a temperature of -20^{o}C until they were needed. The DNA from breeders was obtained from blood samples using the Isolate II Genomic DNA Kit. Subsequently, it was subjected to treatment with RNase A (Bioline, London, UK) according to the instructions provided by the manufacturer.

The genotyping process involved the utilisation of multiplex PCR SMsa-1 and SMsa-2 [24]. The polymerase chain reactions (PCRs) were conducted using an ABI3130 Genetic Analyzer manufactured by Applied Biosystems, located in Foster City, CA, USA. The resulting data was analysed using the Genemapper v3.8 software. Parentage assignment was determined by the exclusion approach using Vitassign v8.2.1 [25]. The gender of the breeder was classified as indeterminate.

4.5. Statistical Analysis

The rates for surface covered by lesions and their intensity were compared using nonparametric tests such as the Wilcoxon test or Kruskal-Wallis test. The data for weight and number of LCDV DNA copies were subjected to a log transformation in order to normalise the distribution. The statistical analysis to assess differences in phenotypes was conducted using Prism8 software (GraphPad, San Diego, CA, USA). The variance components related to the susceptibility to LCDV were separately estimated for batch 1 and batch 2 using bivariate linear mixed models fitted through restricted maximum likelihood (REML) in Wombat (Meyer, 2007). The model used was y = Xβ + Zu + e, where y represents the observed trait, β is the vector for the fixed factor (broodstock origin), u is the vector for the random factor of the animals, and e represents the error. The heritability and correlations between phenotypic and genotypic features were computed for four specific characteristics: weight, length, number of LCDV DNA copies, and LCDV susceptibility. LCDV susceptibility was defined using a binary scale, where 0 represents weak/moderate susceptibility and 1 represents severe/very severe susceptibility, as determined by the SI. Additional runs were conducted using weight as a covariate for SI. However, no discernible impact on genetic estimates was observed, and as a result, it was not included in the analysis.

The heritability estimates for binary LCDV susceptibility were converted from the observed scale to the underlying liability scale using the formula h2 = [p(1- p)/z2] × h2obs, as described by Dempster and Lerner [25]. In this formula, h2obs represents the heritability of the binary trait on the observed scale, h2 represents the heritability on the liability scale, p represents the proportion of affected individuals, and z represents the density of a standard normal distribution at the pth quantile. Furthermore, heritability estimates for the susceptibility of binary LCDV were estimated using a

Bayesian framework. This was done under the threshold model, which was implemented in the MCMCglmm R package [26]. We employed a $\chi 2$ distribution with a single degree of freedom as the previous distribution. Additionally, we utilised a probit scale and set the residual variance to a fixed value of 1. The Markov Chain Monte Carlo (MCMC) algorithm was executed for a total of 1 million iterations, with a thinning interval of 100, following a burn-in period of 100,000 iterations (de Villemereuil et al., 2013). The estimation of heritability for binary traits was calculated using the formula h2 = Va/(Va + 1 + 1), as outlined in the course notes for the MCMCglmm programme [27].

5. Conclusions

This work has developed an optimised system for phenotypically assessing vulnerability to LCDV and ranking animals based on their SI, a composite indicator that combines the extent of lesion coverage and their intensity. The severity categories based on SI showed a strong correlation with the amount of viral DNA in the liver and had a moderate level of heredity. However, these categories were greatly influenced by the age of the animals, suggesting that they could be a useful indicator for selecting animals with a reduced vulnerability to LCDV. The genetic connections between growth and disease features shown a moderate to high level of positivity in younger fish. This suggests the need to construct a combined index for effectively selecting stocks with both reduced susceptibility to LCDV and improved growth.

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