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Estimated genetic parameters and low-density genome scan for SNPs associated with VNN resistance in sea bream

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1 Introduction

Nervous Necrosis Virus (NNV) is one of the major viral pathogens in aquaculture, affecting a wide range of fish species and causing high mortality rates. The gilthead sea bream (*Sparus aurata*) has long been considered resistant to NNV, until recently, when significant mortalities caused by a reassortant NNV strain (RGNNV/SJNNV) were reported in sea bream hatcheries (Volpe et al. 2020). The larval stage is the most susceptible life-stage to NNV, and vaccination is not a feasible option due to the immaturity of the immune system. Selective breeding to enhance resistance against the reassortant NNV strain might be a possibility as a disease preventive action. Here, we analysed for the first time the genetic basis of viral nervous necrosis (VNN) mortality in gilthead sea bream larvae and we assessed the accuracy in the genomic prediction of this trait. Since no information exists on experimental infection of gilthead sea bream with reassortant NNV, several preliminary trials were carried out on sea bream larvae and juveniles at different stages. All preliminary challenges were performed through a collaboration with the Istituto Zooprofilattico Sperimentale delle Venezia (IZSVe), within the framework of an agreement between PerformFISH and MedAID projects. Four experimental trials were performed by infecting sea bream fish of different ages with the RGNNV/SJNNV virus, juveniles of 7g of weight and larvae of 70, 35 and 21 days post hatch (dph). Overall, results showed that the gilthead seabream larvae are susceptible to RGNNV/SJNNV infection irrespective to the age, as proved by the massive viral replication detected by quantitative RT-PCR and the massive presence of immunoprecipitates in the nervous tissues evidenced by immunohistochemistry. However, clear clinical signs and mortality were observed only in the youngest group of 21-dph larvae (Toffan et al. 2021). Therefore, it was decided to perform the challenge to estimate the heritability of resistance to VNN in 20 dph sea bream larvae.

2 Materials and methods

The experimental fish were raised at a commercial hatchery (Panittica Italia) through controlled crosses in three independent full factorial matings (10 sires × 12 dams; 10 sires × 7 dams; 10 sires × 5 dams). At 15 days post-hatching (dph), larvae were transferred to the IZSVe experimental facility. At 19 dph (7 May 2019), larvae were infected by immersion adding to the tank the reassortant strain VNNV/*S.aurata*/Farm1/461-1/Nov2014. The final infectious titre was verified by titration of the water (105.45 TCID50/ml). The challenge trial ended at day 13 post challenge (21 May 2019, see **Figure 1**). Larvae showing symptoms of VNN infection and surviving individuals were collected for DNA analysis and recorded as 0 (asymptomatic) or 1 (symptomatic). The experimental infection protocol was evaluated by the IZSVe Animal Welfare Body and Ethics Committee (Opinion CE.IZSVE.3/2016 of 24/10/216) and subsequently approved by the Italian Ministry of Health (Law decree 101/2017-PR of 02/02/2017).

Due to the small size of the larvae, a DNA extraction test involving the whole larva was performed with three different protocols and kits in order to obtain the greatest amount of high molecular weight DNA: 1) a modified salting out protocol (SSTNE, Pardo et al, 2005), 2) Cells and Tissue DNA Isolation Micro Kit (Norgen Biotek Corp.) in column, and 3) ChargeSwitch® gDNA

Tissue Kits (Invitrogen – Life Technologies) with magnetic beads. Every kit was tested on 8 specimens. The best result for DNA quality and quantity was obtained with the SSTNE method. DNA from parental tissue samples (fins) was extracted with Invisorb Spin Tissue Mini Kit (Invitek Molecular GmbH · D-13125 Berlin). For each sample, DNA quantity was measured with Qubit dsDNA BR Assay Kit (Invitrogen – Life Technologies) and DNA quality was checked on TAE1X buffer 1% agarose gel.

The genotyping was performed using the Axiom SNP Array technology (Thermo Fisher Scientific) at IdentiGEN (Ireland). Eight SSTNE-extracted DNA samples were shipped to IdentiGEN in order to evaluate the use of a lower amount of DNA in comparison with the quantity required by the technology. The genotyping on the eight larval DNA samples using the MedFish SNP array (Peñaloza et al., 2021) provided satisfactory results, so the entire experimental batch and their parents were genotyped using the same strategy. The DNA from 488 symptomatic larvae and 556 survivors, and from the 54 parents, in duplicate, was arranged in nine 96-well plates and shipped to IdentiGEN to be analysed with MedFish SNP array. All the experimental fish and their parents were genotyped using the MedFish SNP array, which contains over 27,000 SNPs for the gilthead sea bream (Peñaloza et al. 2021). Axiom™ Analysis Suite v 5.1.1.1 (Thermo Fisher Scientific) was used for Sample QC analysis and genotype calling from the intensity files using the same software at default parameter values for diploid species (call rate (CR) > 97; dish QC (DQC) > 0.82).

The R package APIS (Auto-Adaptive Parentage Inference Software) version 1.0.1 was used to assign parentage. APIS uses the observed distributions of average Mendelian transmission probabilities to set assignment thresholds, and enables to estimate the proportion of offspring with missing parental genotypes. For all APIS runs, parameters were set to the default values, with the exception of the error rate, which was set to 5%.

Marginal posterior distribution for variance components for VNN symptomatology coded as a binary trait (0 for the fish that were asymptomatics at the end of the NNV challenge test, 1 for the symptomatics ones) were estimated using a Bayesian approach employing Monte-Carlo Markov chain (MCMC) and Gibbs sampling methods, as implemented in the software TM. A single Gibbs chain of 1,000,000 iterations was generated, with a burn-in of 1,000 samples and a thinning interval of 100.

The following univariate sire-dam threshold model was fitted:

$$l_{jkm} = \mu + s_j + d_k + e_{jkm}$$

where l_{jkm} is a latent unobservable variable (liability), μ is the model intercept, s_j is the random additive genetic effect of sire *j*, $d_k$is the random additive genetic effect of dam *k* and e_{jkm} is a random residual.

The median of the marginal posterior density was used as a point estimate for variance components and heritability. Heritability was computed using the sire component only, to avoid potential non-genetic maternal effects, as $h^2 = [4(\sigma_s^2)]/\sigma_p^2$, where σ_s^2 is the sire component of the variance and σ_p^2 is the phenotypic variance. The 95% highest posterior density intervals (HPD95%), the probability for h^2 of being greater than 0.2 and the value of h^2 starting the region of values corresponding to a p = 90% were obtained from the estimated posterior density of h^2 using the R package BOA.

Genome-wide association analysis (GWAS) was performed to test the association between VNN mortality phenotype and SNPs, using the software GCTA (Genome-wide Complex Trait Analysis). A mixed linear model-based association analysis (MLMA) approach was used, following the recommended workflow. Firstly, a genetic relationship matrix (GRM) was calculated using all the 27,706 SNP markers and the result was saved as a binary file containing the lower triangular part of the matrix.
Genomic prediction of VNN mortality was performed implementing three Bayesian regression models: Bayes B (BB), Bayes C (BC) and Bayesian Ridge Regression (BRR; GBLUP equivalent). Prediction performance was assessed by means of five (5) independently-generated 5-fold cross validations (CV). In each CV, 80% of the data were used to train the model and 20% served as a validation set. Three metrics were used to evaluate model performance in classification: Matthews correlation coefficient (MCC), the area under the ROC curve (AUC) and accuracy (ACC). Pedigree indices were estimated using an animal model through 5-fold CV: in each CV, 80% of the data was used to estimate the indices of the remaining 20% of the animals. Performance of the indices in classification of VNN mortality was assessed using the same metrics used for genomic prediction (MCC, AUC, ACC).

3 Results

First symptoms of VNN infection were detected at day 6 post-challenge (13 May 2019), with a sharp peak of mortality. Mortality rapidly decreased and from May 15 afternoon to May 16 in the afternoon no mortality was registered, therefore the greatest part of the larvae was collected and considered as survivors. A limited number of animals was left in the tank to confirm the end of the mortality. No additional mortality was observed and the remaining larvae were collected on May 21 (**Figure 1**). A total of 1,184 individual larvae were collected (513 dying and 671 survivors).

Genomic DNA was extracted from the tissue of 1,044 larvae and 54 parents and used for individual genotyping. A total of 974 larvae, 47% symptomatic and 53% asymptomatic, were successfully genotyped and parentage assignment to a unique parental pair was achieved for all the 974 fish. Individuals were allocated to 160 families, with a number of offspring per family ranging from 1 to 55. After removing one sire and one dam that generated only one offspring, 972 individuals from 28 sires and 22 dams were retained.

Overall, 26,591 SNPs with MAF > 0.05 were scored. The estimate of heritability for VNN mortality was moderate (h2 = 0.1921; 95% highest posterior density intervals: 0.0006, 0.5790), with a probability being greater than 0.2 equal to 0.49. Classification of the observed VNN mortality using the genomic prediction of the phenotype of mortality as classifier was significantly more accurate than random guessing of the classes, with consistent results across Bayesian models (**Table 1**); using the pedigree indices to classify the mortality phenotype resulted in similar performances (AUC = 0.5875, ACC = 0.5798, MCC = 0.2550). **The GWAS failed to identify any genome-wide QTL for VNN mortality overcoming the significance threshold** (**Figure 2**).

VNN is an emerging threat for gilthead sea bream hatcheries and this is the first study that explored the genetic basis of resistance to VNN in this species. Experimental infections in early

developmental stages are scarcely reported, especially with adequate sample size to estimate variance components and genetic parameters. The estimate of heritability for VNN mortality suggests that selective breeding programmes are feasible for increased resistance to VNN of fish larvae/juveniles, overcoming the problem of vaccination. The practical exploitation of genomic information due to the availability of genome-wide dense marker panels might offer the opportunity of developing prediction tools for the studied trait.

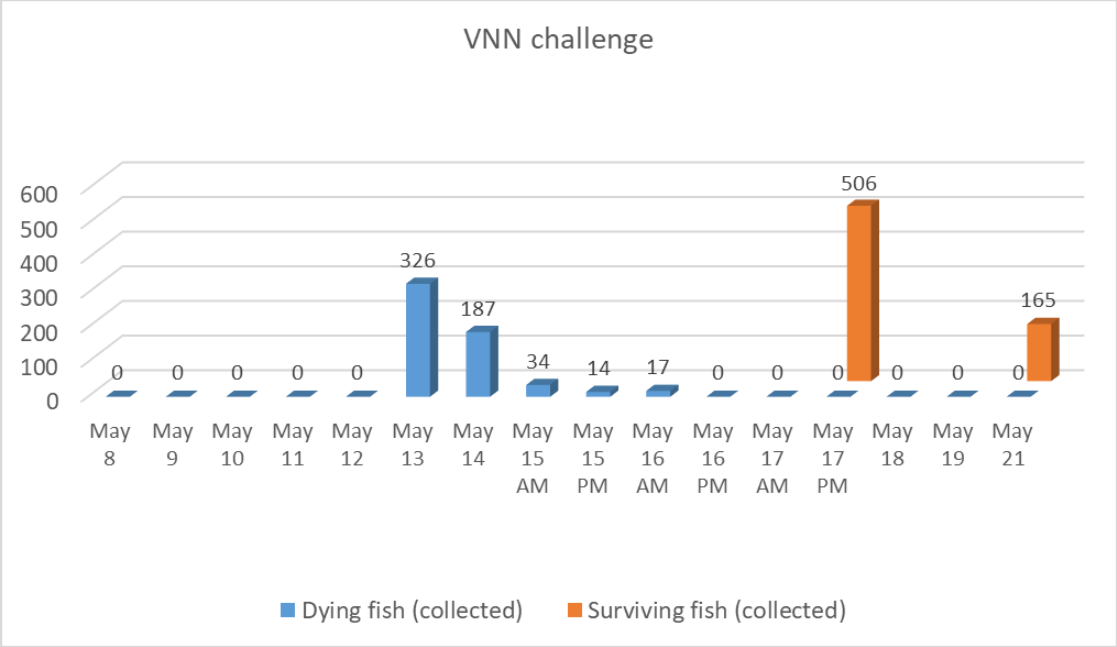

Figure 1. Development of larval mortality post-challenge

Table 1. Average metrics (AUC, ACC, MCC) of classification performance of VNN mortality in 5 independent 5-fold cross-validations; standard deviation (SD) in brackets

Bayesian model	AUC	ACC	MCC
BC	0.5813 (0.0166)	0.5694 (0.0162)	0.1752 (0.0239)
BB	0.5969 (0.0134)	0.5811 (0.0131)	0.1758 (0.0231)
BRR	0.6014 (0.0134)	0.5825 (0.0088)	0.1778 (0.0215)

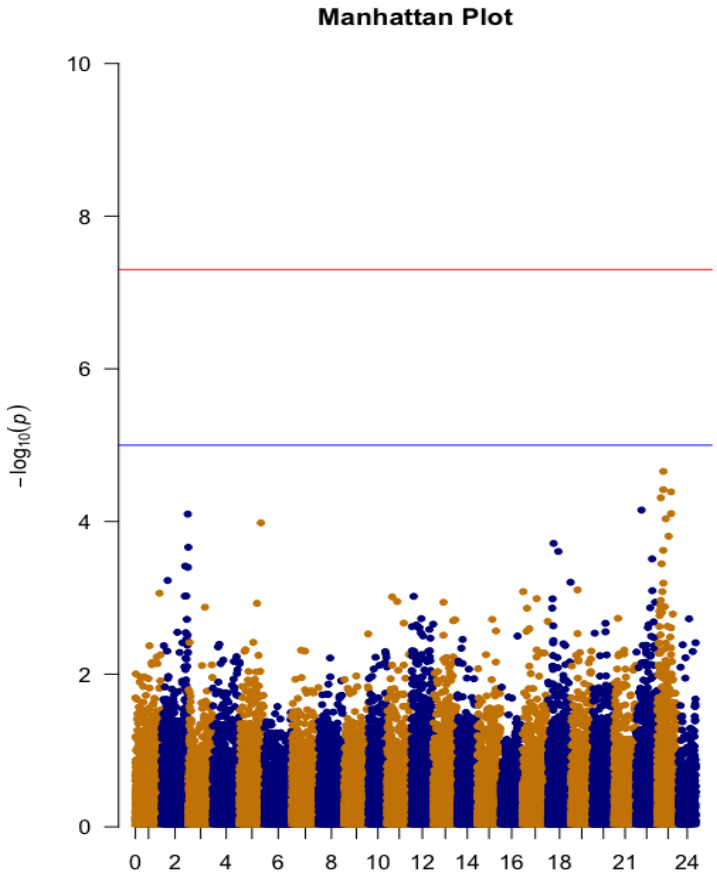

Figure 2. Manhattan plot for GWAS. On the x-axis, SNP position on sea bream chromosomes. On the y-axis, p-values for association of individual SNPs with VNN susceptibility. The blue line corresponds to the chromosome-wide significance threshold, the red line to genome-wide significance.

4 References

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