The presence of the Pacific oyster OsHV-1 resistance marker on chromosome 8 does not impact susceptibility to infection by *Vibrio aestuarianus*

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Running Title: OsHV-1 and Vibrio resistance in oysters

Abstract

The Pacific oyster, *Crassostrea* (*Magallana*) *gigas*, is an important species in global aquaculture, but its production is threatened by pathogens such as ostreid herpesvirus 1 (OsHV-1) and *Vibrio aestuarianus*. A genetic marker for OsHV-1 resistance has been identified on chromosome 8 of the Pacific oyster genome, and selective breeding has been performed to produce oysters with increased survival in response to OsHV-1 infection. However, the potential impact of this marker on susceptibility to other pathogens remains unknown. In this study, we assessed the effect of the presence and allelic dosage of the OsHV-1 resistance marker on the susceptibility of selectively bred juvenile oysters from the Molluscan Broodstock Program of Oregon State University to *V. aestuarianus* infection. Sixteen families were produced with various genotypic states of the marker, and a disease challenge with *V. aestuarianus* was performed on these families to track individual mortality. Challenged oysters were genotyped at the OsHV-1 resistance locus, and *Vibrio* susceptibility was compared among genotype groups within and between families. Additionally, reduced-representation sequencing was performed on a subset of challenged oysters to conduct a genome-wide association study for *Vibrio* survivorship. Before *Vibrio* exposure, the OsHV-1 resistance marker was not observed at the expected Mendelian ratios in families with heterozygous parents; a significant reduction in the homozygous alternate genotype was observed in multiple families. Mortality rates differed significantly among families in the *V. aestuarianus* challenge (range: 47.9% to 85.4%), but no association between the OsHV-1 resistance marker and survival to *Vibrio* infection was observed. Additionally, no loci were significantly associated with *Vibrio* survivorship in these samples, suggesting polygenic trait architecture.

Keywords: genome-wide association study; OsHV-1; ostreid herpesvirus 1; Pacific oyster; *Vibrio aestuarianus*; virus.

Introduction

The Pacific oyster *Crassostrea* (*Magallana*) *gigas* plays a prominent role in the aquaculture industry, accounting for more than 98% of all oyster production worldwide, with over five million metric tons produced annually (Azéma et al., 2017; Botta et al., 2020; Laurence et al., 2009; Okon et al., 2023). Native to the northwest Pacific Ocean, *C. gigas* has been introduced globally for aquaculture purposes because of its high growth rate and adaptability to various environments (Zhai et al., 2021). A goal of the aquaculture industry is to supply aquatic-sourced proteins to meet human dietary requirements for the rapidly growing global population (Yang et al., 2022). However, the aquaculture industry faces substantial challenges owing to pathogenic infections and outbreaks caused by viruses and bacteria (Yang et al., 2022). Since 2008, there has been a global increase in oyster mass mortality events, which has substantially decreased *C. gigas* production in Europe and Australia (Barbosa-Solomieu et al., 2015; Fuhrmann et al., 2019). For example, Pacific oyster production in New South Wales (Australia) decreased by 48% within three years of the first OsHV-1 outbreak. Decreases in production can cause significant increases in prices. In France, the price of oysters increased from € 4.93/kg in 2001 to € 7.78/kg in 2012, when the surviving spat from a 2008 mortality event reached market size (Fuhrmann et al., 2019). Given that oysters possess only innate immunity, resistance to pathogens cannot be enhanced long-term through vaccination, but rather by methods such as immunostimulation, diet, and genetic selection (Divilov et al., 2021; Robinson & Green, 2020; Yang et al., 2022). Furthermore, oysters are typically cultivated in open environments within straits and bays, with complete contact with flow-through seawater, making it impossible to fully control diseases through the administration of disinfectants, antibiotics, or similar treatments. Quarantining oysters in open environments is not feasible; therefore, large-scale pathogen outbreaks are likely to occur. Genetic improvement through selective breeding is considered to be the most practical approach for enhancing disease tolerance and resistance (Green et al., 2023; Zhai et al., 2021).

Ostreid herpesvirus 1 (OsHV-1) is a pathogen responsible for large-scale mortality events in Pacific oysters. In 2008, a highly virulent form of OsHV-1 termed OsHV-1 microvariant (µVar), was detected in France (Segarra et al., 2010). Outbreaks of OsHV-1 µVars have subsequently occurred in Italy, the United Kingdom, Australia, New Zealand and the United States (de Lorgeril et al., 2018; Divilov et al., 2023b; Government of Canada, 2018). OsHV-1 affects oyster immunity and allows opportunistic bacteria, such as *Vibrio* species, to infect hosts (Siboni et al., 2024). OsHV-1 can infect all oyster life stages, especially larval and juvenile stages, resulting in up to 100% mortality (de Lorgeril et al., 2018). Therefore, substantial efforts have been made to limit the spread of OsHV-1. In 2018, a novel OsHV-1 µVar was detected in San Diego Bay, California, USA, posing a significant risk to oyster aquaculture on the Pacific west coast of North America (Burge et al., 2021; Thompson et al., 2024). Recent research using high-throughput sequencing

indicated that this strain is different from the µVar detected in France with a 6 bp deletion in the microsatellite region instead of a 12 bp deletion, in addition to other differences (Pelletier, 2023). Given the threat posed by OsHV-1, substantial efforts have been made to implement strategies to mitigate OsHV-1 spread.

Extensive efforts in selective breeding programs have improved the OsHV-1 survivability of Pacific oysters in the USA and Australia as a potential mitigation strategy against OsHV-1 related mortality (Divilov et al., 2023b; Kube et al., 2018). A quantitative trait locus (QTL) has been associated with an upregulated interferon regulatory factor (IRF) antiviral gene (i.e., *irf2*) on chromosome 8, which is thought to increase resistance to OsHV-1 infections (Divilov et al., 2021). Oysters with the alternate allele of this locus may be genetically primed to defend against viral infections. This marker has been used in marker-assisted selection using a rhAmp genotyping assay (Beltz et al., 2018; Divilov et al., 2023b). The rhAmp genotyping assay is a fluorescence-based PCR method that allows rapid and efficient SNP genotyping of specific alleles at a locus. Selectively bred families that carry the OsHV-1 resistance marker were produced by the Molluscan Broodstock Program (MBP) of Oregon State University. However, selective breeding may lead to fitness trade-offs (Divilov et al., 2024). For example, increased OsHV-1 resilience in offspring of oysters primed with an OsHV-1 viral mimic has been reported to co-occur with higher *Vibrio* infection levels in offspring (Robinson & Green, 2020). OsHV-1 resilience was also correlated with decreased larval size and altered microbiome composition. This raises the question of whether virally resistant oysters are more susceptible to bacterial pathogens, such as *Vibrio aestuarianus*.

Bacteria from the genus *Vibrio* are prevalent in marine environments, and several pathogenic strains threaten vertebrates and invertebrates (Wendling et al., 2014). *Vibrio* species can utilize a variety of different niches, and include forms that are free-living, mutualistic, opportunistic, or pathogenic. Some *Vibrio* spp. can result in vibriosis, a disease that causes mass oyster mortalities. Common species of *Vibrio* that are pathogenic to oysters include *V. splendidus*, *V. anguillarum*, *V. tubiashii* and *V. aestuarianus* (Wendling et al., 2014). In Europe, *V. aestuarianus* has caused recurrent mortalities since 2001, first in France and then in other European countries, including Italy, Ireland, and Spain (Mesnil et al., 2023). In recent years, the frequency of mortality events in France has increased from 30% in 2011 to 77% in 2013, when it has emerged as the primary pathogen identified during summer mortality events (Coyle et al., 2023). In British Columbia (BC), Canada, *V. aestuarianus* is one of the most common pathogenic *Vibrio* species and is emerging as a serious threat to oyster aquaculture (Cowan et al., 2024). Considering the potential for marker-assisted selection using the OsHV-1 resistance marker in the Pacific Northwest and given that the effects of the OsHV-1 resistance allele on *Vibrio* resistance remain unknown, further investigation is required. Furthermore, loci associated with increased survival to *V. alginolyticus* in Pacific oysters have

been identified (Yang et al., 2022), and if they are identifiable in response to *V. aestuarianus*, they would be beneficial for selective breeding purposes.

In the present study, we evaluated the susceptibility of Pacific oyster juveniles selectively bred for OsHV-1 resistance to *V. aestuarianus* infection by examining the effects of the presence and allelic dosage of the OsHV-1 resistance allele on survival to *Vibrio* challenge. Given the functional differences in immunological responses to viruses and bacteria (Green et al., 2016), we hypothesized that there may be immunological trade-offs between resistance to viral and bacterial pathogens. Additionally, using oysters challenged with *Vibrio* from the four MBP families, we performed double digest RAD-sequencing (Peterson et al., 2012) to investigate associations of loci with *Vibrio* resistance. This study provides insight into how genetic predispositions towards resistance against one viral pathogen (OsHV-1) in a shellfish species may affect its vulnerability to a bacterial pathogen, specifically *V. aestuarianus*.

Methods

Samples and crosses

Pacific oyster families with various genotypic states of the chromosome 8 (Chr8) resistant marker (i.e. position 9,719,736 on Chr8; Divilov et al., 2023) were generated by Oregon State University's Molluscan Broodstock Program (MBP; Oregon, USA). Spat from 16 of these families were provided to Vancouver Island University (VIU) to be used in experimental exposures to *V. aestuarianus*. Spat were 18 weeks old, greater than 4 mm long, and less than 6 mm wide. Animals were originally transported to the Deep Bay Marine Field Station (VIU; British Columbia, Canada), where they were placed overnight in 12.5 ^{o}C flow-through seawater tanks, after which they were transported to the Center for Shellfish Research (CSR) at the VIU campus in Nanaimo, BC. Seawater and algae (*Chaetoceros muelleri*) samples were collected from Deep Bay Marine Field Station and transported to VIU.

Four of the 16 families were designated as 'mapping families' (i.e., F114-F117) based on the genotypic state of the parents at the Chr8 marker (i.e., both parents were heterozygous). The other 12 families were designated as 'non-mapping families,' which had at least one homozygous reference parent (F101-F103, F105-F113; Table S1). None of the families were produced by crosses in which both parents were homozygous for the alternate allele (i.e., the allele that confers resistance). Additionally, one family from the VIU oyster breeding program (Oyster Family Run 6, family 10; OFR6-10) was also included in the *Vibrio* exposure, and this family had not been selected for OsHV-1 resistance.

Disease challenge with Vibrio aestuarianus

A disease challenge was performed using *V. aestuarianus* to determine whether oysters with different genotypic states of the OsHV-1 resistance locus were differentially susceptible to infection by *V. aestuarianus*. Forty-eight spat from each of the 16 MBP families and the VIU family (total n=768) were exposed to *V. aestuarianus*. An additional mixture of samples from multiple families was treated in the same way as the exposed oysters but without exposure to *Vibrio* and was therefore used as a non-exposed control group (n = 48 animals). For the exposure, spat were placed in 12 well plates, where each well contained 3 mL seawater and one spat. Spat in plates were acclimatized overnight in a temperature-controlled room at 16 ºC.

A *Vibrio* suspension was prepared by inoculating *V. aestuarianus* (strain: BS032 2018) in two flasks containing TSB + 2% NaCl overnight in an orbital shaker (23 ºC and 200 rpm). The cultures were then removed from the incubator, transferred into Falcon tubes, and centrifuged (5000 x g) at ambient temperature for 5 min. The supernatant was discarded and the cell pellets were resuspended in seawater. The optical density of the resuspended cells was measured at 600 nm using an Eppendorf BioSpectrometer on an Eppendorf µCuvette® G1.0. A standard curve was prepared to estimate the amount of *V. aestuarianus* in the preparation. The concentration of the *Vibrio aestuarianus* inoculum was also estimated by serial dilution and plating on tryptone soy agar with 2% NaCl. Colonies were enumerated after incubation at 23 ºC for 48 h.

The exposure trial was initiated by adding 300 µL of the *V. aestuarianus* suspension (i.e., 7.35×10^7 total cells) to each well on each experimental plate. Sterile seawater was added to the control plate. The temperature of the room was then increased to 23 ºC for five days to simulate a marine heatwave event, and the temperature was reduced to 16 ºC on the sixth day of exposure (Green et al., 2019). After *Vibrio* inoculation, 100 µL of algae (*C. muelleri*) was added to each well of all plates, including the control plate. Morbidity was measured daily for six days, after which time the disease challenge ended. Oysters were considered moribund or deceased when their shell valves were gaping and failed to close after gently touching the oysters with a sterile toothpick. Oysters were placed into 25 mL Falcon tubes containing 100% ethanol, where whole individuals were pooled in the storage tube depending on their day of death and family. After six days, surviving oysters were preserved and stored in 100% ethanol (as described for dead oysters). The ethanol-preserved oysters were stored at room temperature until DNA extraction. Mortality data were analyzed using IBM SPSS Statistics v28.0.1.0 (142) (IBM Corp., Armonk, NY) and R (R Core Team, 2025). Mortality distributions were assessed using a Kaplan-Meier survival analysis. A log-rank test was conducted to determine the differences in survival distributions among families, and a pairwise log-rank test was performed to determine which families had different survival distributions.

Genomic DNA extraction

DNA extractions were performed on oysters from the mapping families (i.e., F114-F117) to extract total genomic DNA using a Monarch® Genomic DNA Purification Kit (#T3010L) according to manufacturer instructions for extractions from tissue samples. Briefly, oyster spat samples were extracted from their shells using sterilized forceps and rinsed with deionized water to remove residual ethanol. The whole spat tissue was then placed in a digestive solution containing lysis buffer and proteinase K (NEB). Samples were homogenized using a sterile pestle and digested at 56 ºC for 2 h, after which an RNase incubation was performed. Genomic DNA was purified through an extraction column as per manufacturer's guidelines and eluted in 35 µL molecular-grade water held at 60 ºC. DNA quantification was performed via absorbance and Qubit fluorimetry using an Eppendorf BioSpectrometer on an Eppendorf µCuvette® G1.0. Samples were checked for purity using the A260/A280 and A260/A230 ratios and quality using 1% agarose gel electrophoresis (see Supplemental Information). Extracted genomic DNA was then stored at -20 ºC.

OsHV-1 resistance allele dosage by rhAmp SNP genotyping assay

The rhAmp SNP assay (Design ID: CD.GT.ZKJS3200.1; Integrated DNA Technologies) targeting a G/A (ref/alt) SNP at position 9,719,736 on Chr8 (Divilov et al., 2023b) was used to genotype the OsHV-1 resistance marker in all exposed individuals in the mapping families (F114-F117). The assay was conducted on a CFX96 Touch Real-Time PCR Detection System (BIO-RAD) using the manufacturer's instructions for a 5 µL reaction volume in 96-well plates (Table S2). Samples were normalized to 10 ng in 2 µL molecular water (5 ng/µl), and each sample was run in duplicate. Negative controls were also run on each plate, containing only molecular-grade water. The assay was previously confirmed to work on gBlocks as per manufacturer's recommendations, and samples of known genotypes were used as controls in the study. The fluorophore dyes FAM and VIC were used for genotyping the reference and alternate alleles, respectively, and to call genotypes as homozygous reference, heterozygous, or homozygous alternate.

Results of the rhAmp assay were exported from the instrument in text format and processed in R v4.3.2 using a custom script (see *Data Availability*). As some likely false positives were observed, where one dye was detected at a much later Cq value than the other detected dye, a correction was applied to convert the suspected false-positive heterozygote to a homozygote. Specifically, Cq differences between FAM and VIC fluorophores were calculated per well, and any cases where the absolute value of the Cq difference between the two fluorophores was greater than four, the call was determined to be a false positive

for the later Cq fluorophore, which was then set to zero, making the call a homozygote for the earlier fluorophore (Figure S1 and Figure S2). Using the corrected data, genotypes were assigned based on the retained fluorophore calls: FAM only = homozygous reference; VIC only = homozygous alternate; both FAM and VIC = heterozygote; no fluorophore call = missing. Technical reproducibility was evaluated at the genotype level (Table S3), and the final call for technical replicates was based on the majority call, as inconsistent calls were re-run. The genotyping results were plotted and analyzed in R (see *Data Availability*).

Statistical analysis of Chr8 genotypes was carried out using IBM SPSS Statistics v28.0.1.0 (142) and R. The effect of the Chr8 marker genotype on mortality from the *Vibrio* challenge was analyzed using a chi-square test of homogeneity (or Fisher's exact test if the chi-square assumptions could not be met). To use the chi-square test, samples had to meet the following criteria: one dependent variable measured at the dichotomous level (mortality: dead vs. alive or time until death), one independent variable with three or more categorical groups (genotype: *AA, Aa, aa*), and independence of observations. Finally, the sample had to meet a minimal sample size requirement. Minimal sample size with respect to expected frequencies was required to have an expected count greater than or equal to five. If samples did not meet this last criterion, a Fisher's exact test was performed instead of the chi-squared test. Genotype frequencies in each family of the four mapping families (i.e., with both parents heterozygous for the Chr8 locus), independent of mortality phenotype, were assessed using the same method to determine whether there were significant differences in genotype frequencies across families prior to the *Vibrio* challenge.

Double-digest RAD sequencing (ddRADseq) sample preparation and read processing

Genomic DNA from the OSU mapping families (i.e., F114-F117) and their respective parents, and OFR6 Family 10 from the VIU breeding program were used for double-digest restriction site-associated DNA sequencing (ddRADseq), which was carried out at the Institut de Biologie Intégrative et des Systems (IBIS) at Laval University, Quebec, Canada, using *Nsi*I/ *Msp*I as cutting enzymes as previously described (Sutherland et al., 2020). Samples were pooled with 32 individuals per sequencing run and sequenced on an Ion Torrent Proton using a ThermoFisher Ion PI Chip Kit v3 chip, as per manufacturer's instructions. Base calling was performed using Torrent Suite 5.10.1 (ThermoFisher), and multiplexed FASTQ output files with barcodes still present were exported using the Torrent FileExporter plugin.

Sequence data processing and analysis were conducted using the repository *stacks_workflow* (Normandeau, 2013/2025) and Stacks v.2.3e (Rochette et al., 2019), accompanied by custom analysis scripts (see *Data Availability*). The quality of all FASTQ files was assessed before and after trimming using

FastQC v0.11.8, and the results were consolidated using MultiQC (Andrews, 2010; Ewels et al., 2016). To prepare the data, universal adapters were eliminated and reads longer than 50 bp were retained using cutadapt v1.18 run in parallel (Martin, 2011; Tange, 2015). Quality trimming was performed on all reads with Phred >10 in sliding windows of size 15% of the read length, followed by truncation to 80 bp and demultiplexing using the *process_radtags* function of Stacks (Catchen et al., 2011; Rochette et al., 2019), with two enzymes selected (i.e., *Nsi*I and *Msp*I) in parallel.

Demultiplexed and trimmed single-end reads per individual were aligned to a chromosome-level genome assembly for Pacific oysters (i.e., GCF_902806645.1_cgigas_uk_roslin_v1_genomic.fna) (Peñaloza et al., 2021) using bwa mem v0.7.12-r1039 (Li et al., 2009; Peñaloza et al., 2021). Samtools v1.9 was used to remove unmapped reads and secondary or low-quality alignments (Li et al., 2009). The remaining reads following quality control were genotyped using Stacks. The number of reads and alignments per sample, as well as alignment rates were calculated using samtools and custom scripts.

Loci were identified with Stacks using the reference-based approach through the *gstacks* module with default settings. Genotype data were filtered using the *populations* module of Stacks, requiring that a locus be genotyped in at least 70% of the individuals in all six populations (i.e., F114-F117, MBP parents, and OFR6.10), and with a minimum global minor allele frequency (MAF) of 0.01. Samples with fewer than 1 M reads were identified and removed from the analysis, as previously described (Sutherland et al., 2020). Samples were genotyped again using *gstacks* as described above, and outputs were used for downstream analysis. Data were exported including a single SNP per RAD-tag data using the *populations* module as a VCF file. The VCF file was imported into R using vcfR (Knaus & Grünwald, 2017) and converted to genind format for use with adegenet functions (Jombart & Ahmed, 2011). Functions from the *simple_pop_stats* repository were employed for analysis (see *Data availability*). The genotyping rate per sample was determined using the *percent_missing_by_ind* function from *simple_pop_stats*, retaining individuals with less than 30% missing data. Minor allele frequency (MAF) was re-calculated per locus, and loci with MAF < 0.01 were excluded. H_{OBS} and Hardy–Weinberg (HW) equilibrium filters were not applied due to anticipated family effects. A principal components analysis (PCA) based on genotypes was conducted using the *glPca* function of adegenet.

Genome-wide association study (GWAS) for Vibrio survivorship

A GWAS for survivorship from *Vibrio* exposure was conducted using GEMMA v0.98.5 (Zhou & Stephens, 2012) using the single-SNP per locus VCF file, as described above, prepared in GEMMA formats in R (see *Data Availability*). NCBI chromosome identifiers were converted to linkage groups using the Pacific oyster

assembly *Cgigas_uk_roslin_v1,* and linkage groups were converted to chromosomes following Peñaloza et al. (2021). A GWAS analysis was conducted using unimputed genotypes, as well as using genotypes that were imputed per family using the *impute_mean* function of the *missMethods* R package (Rockel, 2022). Only MBP families were used for GWAS, excluding the VIU family. The GEMMA analyses were conducted on all MBP families together by calculating the kinship between samples, using either a binary phenotype (dead or alive) or a phenotype of days-to-death, where survivors of the trial were given the value of day 7, or one day after the trial concluded. A Bonferroni-corrected *p*-value of 0.05 was used as the genome-wide significance threshold. SNPs were coded 0, 1, and 2 to correspond to homozygote reference, heterozygote, and homozygote alternate genotypes, respectively. The R package *fastman* (Paria et al., 2022) was used to generate Manhattan plots from the GWAS analysis.

Results

Initial genotype frequencies of OsHV-1 resistance marker

Based on all selectively bred oysters in the exposure trial (n = 48 per family), the Chr8 marker genotype frequencies per mapping family (i.e., F114-F117) differed slightly from the expected proportions based on Mendelian inheritance from the heterozygous parents (i.e., 1:2:1 for homozygous reference, heterozygous, and homozygous alternate). Heterozygotes were the most frequently observed genotype across all families (57%), followed by the homozygous reference (25%), and the homozygous alternate genotypic state was the rarest (18%; Figure 1). When inspecting individual families, a skew was observed where family F115 had significantly more homozygous alternate genotypes (35%) than expected, whereas families F114 and F117 had significantly fewer (11% and 7%, respectively; χ^2 test of homogeneity $p<0.05$). No other genotypes were significantly different among the families (Table S4).

Mortality in Vibrio aestuarianus *disease challenge*

Mortalities were tracked daily following the exposure to *V. aestuarianus* during the disease challenge experiment. No mortality was observed in the first two days post-exposure (DPE). Considering all families together, 37.8% of oysters died on 3 DPE, and by 6 DPE 67.2% of all exposed oysters had died (n = 516) (Figure S3). The remaining 252 *Vibrio*-exposed oysters (32.8%) survived the disease challenge.

Considering cumulative mortality per family, the mortality rate during the disease challenge was on average (± s.d.) 67.2 ± 9.5% (Figure 2). Family F106 exhibited the highest mortality (85.4%), and family F116 exhibited the lowest mortality (47.9%). Rates of mortality also varied among families ($p<0.001$; Figure 3), and pairwise differences were observed between family F106 and families F103, F113, F114,

F115, and F116 ($p < 0.003$; Table S5; Bonferroni correction significance threshold $p = 0.003$). The offspring of the family with the highest and most rapid mortality (i.e., F106) were expected to be completely heterozygous for the Chr8 marker. The offspring of the families with the lowest mortality (i.e., F103 and F113) were expected to all be homozygous for the reference allele (Table S1). However, families grouped by cross-type (i.e., genotypic state of parents at the Chr8 marker) did not show a consistent overall mortality trend (Figure 2). The mixed-family control group, unexposed to *Vibrio*, exhibited 100% survival as expected.

Effects of OsHV-1 Chr8 resistance allele on survival to Vibrio challenge

The effects of the presence and dosage of the Chr8 resistance allele on mortality for families F114-F117 were evaluated based on days to death (Figure 4) or survival (dead or alive; Figure 5). No significant associations were observed between offspring genotype and mortality for any of the families considering days to death, nor dead or alive ($p>0.05$). The analysis was conducted for each family individually to avoid potential confounding interfamily effects.

ddRAD-seq genotyping of Vibrio exposed families

Oysters from families F114-F117 (i.e., MBP families) and OFR6.10 (VIU family) that were exposed to the *Vibrio* challenge were sequenced by ddRADseq (avg. per family = 36.4 individuals). Additionally, the parents of MBP families were sequenced (Table S6). On average, 92.8 M single-end reads were provided per chip from the six chips sequenced (n = 523.1 M reads total). Demultiplexing resulted in an average of 91.8% of reads retained. On average (± s.d.), 65.0 ± 2.2% of reads aligned against the chromosome-level reference genome (Peñaloza et al., 2021) and 340,291,536 alignments were used for genotyping with *gstacks*. In total, 277.6 M primary alignments (81.6%) were retained (10.9% of alignments were discarded owing to insufficient MAPQ and 7.5% due to excessive soft clipping). On average, 1.79 M alignments were obtained per sample (range: 0.265-4.681 M records per sample). Samples with fewer than 1 M reads were removed, resulting in the removal of 18 of the 190 total samples, leaving 172 remaining samples (Table S6). Notably, 15 of the 18 removed samples were oysters that died during the trial, suggesting that these samples may have had DNA degradation occurring between the mortality event and sampling, which has also seen in other challenge studies for Pacific oysters (Sutherland et al., 2025).

After low-yield samples were removed, genotyping used 330.6 M alignments (269.7 M primary alignments) to build 393,153 RAD loci with an average (± s.d.) effective coverage of 56.9 ± 22.6x. Loci

were filtered to retain those that were genotyped in at least 70% of the individuals in all families and with a minor allele frequency (MAF) > 0.01. This resulted in the retention of 10,202 RAD loci comprising 889,291 genomic sites, of which 25,801 contained variants (percent polymorphic = 2.9%). Considering only a single SNP per RAD locus resulted in 8,252 variants that were retained. Individuals missing more than 30% of loci were removed, leaving 168 retained individuals (Figure S4; Table S6). This included the retention of 32, 36, 35, 33, and 25 individuals from F114, F115, F116, F117, and OFR6.10, respectively, as well as seven of the eight parents of families F114-F117. Reapplying the MAF filter after individuals with low genotyping rates were removed resulted in a total of 8,193 SNPs retained, of which 4,218 were MAF > 0.1. Visualizing the samples clustered by PCA based on genotypes showed close clustering of samples within the four MBP families, with the parents of these families interspersed among the offspring and the VIU family grouping together but apart from the MBP families on PC1 (Figure S5). One individual from F116 clustered within the F115 family, suggesting a sample labelling or handling error (Figure S5).

Genome-wide association study for survival to Vibrio challenge

Selectively bred oysters from MBP families F114-F117 were used for GWAS analyses considering survival to the *Vibrio* challenge as a dichotomous variable (dead or alive) or as days-to-death. This included 49 oysters that survived and 87 that died (n = 136). Considering only loci on chromosomes (n = 7,870 SNPs), without imputation 3,656 SNPs were analyzed by GEMMA with a MAF > 0.05 filter applied. The percent variation explained (PVE) ± standard error (S.E.) was 19.2 ± 14.7% for survival as a binary trait and 6.3 ± 9.8% for days post-exposure survival. There were no significantly associated loci to survivorship to the *Vibrio* challenge considering survival as dead or alive, or as days-to-death (Figure 6). Similarly, no significant associations were identified using the day-to-death phenotype (Figure S6A). Using mean imputed genotypes, 5,478 of the 7,870 chromosomal SNPs were analyzed by GEMMA (MAF > 0.05), resulting in similar PVE values (PVE ± S.E. for dead/alive variable = 18.1 ± 14.8% and for days-to-death = 5.7 ± 9.6%), and also did not identify any loci significantly associated with the phenotype survival or days-to-death (Figure S6B and Figure S6C).

Discussion

Genetic and epigenetic factors of hosts are known to influence disease interactions (Krickeberg et al., 2012; Magwire et al., 2012). In this study, we assessed whether the presence of a viral resistance marker affects host susceptibility to a bacterial pathogen. Our results indicate that the presence and allelic dosage of the

OsHV-1 resistance marker (*i.e.*, the Chr8 marker) (Divilov et al., 2023b) in selectively bred *C. gigas* does not affect the susceptibility of juvenile oysters to *Vibrio aestuarianus* infection. Juvenile Pacific oysters have previously been shown to be susceptible to *V. aestuarianus* in BC, Canada (Cowan et al., 2024). These results provide important insights into selectively breeding for disease resistance in Pacific oysters. This may also suggest an absence of genetic correlation between OsHV-1 resistance and *V. aestuarianus*, at least regarding the OsHV-1 resistance mechanism associated with the Chr8 resistance marker. Given the independence observed, identifying loci involved in resistance to *V. aestuarianus* may result in the ability to select oysters resistant to both OsHV-1 and *V. aestuarianus*, an outcome that would bring significant benefits to the field. However, we were not able to identify any major effect loci for resistance to *V. aestuarianus* and rather indicated that the differences in susceptibility observed may be controlled by polygenic architecture.

Effects of the OsHV-1 resistance allele on survival to Vibrio aestuarianus *infection*

The presence of the OsHV-1 resistance allele had no effect on the survival to *V. aestuarianus* infection. This finding is supported by another study that reported the absence of genetic correlations between OsHV-1 and *V. aestuarianus* resistance (Azéma et al., 2017). Azéma et al. (2017) analyzed the genetic parameters for resistance to OsHV-1 and *V. aestuarianus* at three different life stages: spat (3 to 6 months old); juvenile (11 to 15 months old); and adult (25 to 26 months old). They reported no observable genetic correlation between survival after viral infection and survival after bacterial infection. However, the life stage of the oyster significantly affected susceptibility to both pathogens; susceptibility to OsHV-1 decreased with age and growth, whereas susceptibility to *V. aestuarianus* increased with age and growth. The different effects of age on susceptibility to OsHV-1 and *Vibrio* may reflect the different immune responses of oyster hosts. Further evidence of the independence of the resistance mechanisms was provided by Green et al. (2016), who found different transcriptome responses to OsHV-1 and *V. aestuarianus* infections. Thus, the immune genes involved in the defence against OsHV-1 and *Vibrio aestuarianus* infections appear to be distinct.

The absence of a correlation between the OsHV-1 resistance marker genotype and *Vibrio* susceptibility suggests that the mechanisms of resistance to these pathogens are independent. The OsHV-1 resistance allele is thought to upregulate *interferon regulatory factor 2* (*irf2*), an antiviral gene that can provide oysters with constitutively upregulated antiviral immunity (Divilov et al., 2023b). With constitutively overexpressed *irf2*, overexpression of antiviral genes could be expected even in the absence of the virus. Therefore, the lack of increased susceptibility to *Vibrio* is notable, as it suggests that the upregulated antiviral immunity does not negatively or positively impact the oyster's ability to resist

bacterial infections. The genetic mechanisms underlying *Vibrio* resistance are not well known; however, a recent study using 52 Pacific oyster families found 18 SNPs in three QTL associated with resistance to *V. alginolyticus* in *C. gigas,* and the authors suggested that *Vibrio* resistance is polygenic (Yang et al., 2022). Most of these genes are related to pathogen recognition and immune defence responses (Yang et al., 2022). Collectively, genes that induce the expression of immune-related genes are potential targets for selective breeding to increase *Vibrio* resilience.

Selective breeding for one trait can lead to fitness trade-offs by negatively affecting a second trait and consequently impacting overall fitness (Divilov et al., 2024; Dwivedi et al., 2021).Our understanding of the compromises in selection for disease resistance is limited, particularly regarding the effects on other traits or resistance to other pathogens. Trade-offs can arise when beneficial traits are linked to detrimental ones, thereby affecting fitness outcomes (Gallardo-Hidalgo et al., 2021). For example, although selection for increased growth rate has also improved larval resilience to ocean acidification, fast-growing oysters later in life are more susceptible to marine heatwaves and *V. aestuarianus* infections; mortality was found to increase by 76% per gram of body weight gained (Khtikian, 2021; Nordio et al., 2021). However, selection for OsHV-1 resistance in the MBP breeding population has not been observed to impact growth (Divilov et al., 2024). Selection for disease resistance is complex, as resistance is expected to be influenced by multifactorial biological functions, including reproduction and immunity (Bai & Plastow, 2022; Wang et al., 2021).

We expected a trade-off may be observed in the present study because up-regulation of the immune system can increase the energy requirements of the host (Lochmiller & Deerenberg, 2000; Robinson & Green, 2020). This can reduce the overall reproductive success, growth rates, and survival to events such as pathogen outbreaks (Lochmiller & Deerenberg, 2000; Robinson & Green, 2020). Additionally, in some cases, over-reactive immune responses to pathogens can be more damaging than a weak immune response (Green et al., 2016), as discussed in the concept of immune tolerance (Råberg et al., 2007). The expression of extremely potent antibacterial or antiviral effectors can result in a toxic cellular environment that can be detrimental to the organism (Green et al., 2016), and strong immune effectors and responses can inflict damage on host tissues. In the case of infectious diseases, a fitness trade-off might occur if the heightened immune response leads to greater collateral damage (Råberg et al., 2007). Furthermore, other environmental stress factors may impact immune responses due to energetic constraints (Sutherland et al., 2014). Here, we did not measure other factors that may represent energetic trade-offs for increased viral resistance (e.g., growth rate and response to other stressors), but these factors could be integrated into future studies to evaluate the impact of the OsHV-1 marker on *Vibrio* resistance. Notably, before the *Vibrio* exposure occurred, there were baseline differences in the genotype frequencies of the Chr8 marker, specifically in

the proportion of the homozygous alternate genotype. Therefore, other unknown factors may cause distortions in the frequency of this marker, for example, the presence of multiple linked QTL on Chr8 (Divilov et al., 2023a). Other distorting factors could affect future breeding practices around the Chr8 marker.

Although we did not observe a trade-off, selection for disease resistance may also indirectly increase resistance to other stresses such as marine heat waves (MHW). MHWs increase water temperature, resulting in high mortality rates (Cowan et al., 2024). Vibriosis and *V. aestuarianus* proliferation are further linked to seawater temperature, with increased temperature increasing host susceptibility by weakening the immune system and increasing the transmission and proliferation of pathogenic *Vibrio* species (Cowan et al., 2024; Wendling et al., 2014). MHWs are a consequence of climate change, which is driven by increased anthropogenic emissions of CO_2. The global average MHW frequency and duration increased by 34% and 17%, respectively, resulting in a 54% increase in annual marine heatwave days globally (Oliver et al., 2018). Bolstering resilience to MHWs may be achieved by producing oysters with increased resistance to viral and bacterial pathogens. This can mitigate the detrimental effects of these temperature spikes on oyster populations. This dual-pathogen resistance strategy could serve as a multifaceted approach to bolster oyster health and ensure the sustainability of oyster aquaculture in the presence of various environmental stressors.

Family effects and GWAS for V. aestuarianus *resistance*

In this study, we also analyzed family differences in survival following *Vibrio* infection, both in cumulative mortality and mortality rates (days to death). Although different families have known OsHV-1 genotypes, they also would have other genetic differences that could affect resistance to *V. aestuarianus*. As the OsHV-1 resistance marker did not explain the variance observed across families in *Vibrio* susceptibility, other factors may be involved.

In the GWAS analysis for survivorship to *V. aestuarianus*, we did not identify any loci significantly associated with mortality or mortality rate in response to infection. It is possible that our sample size was too small to detect a more subtle genetic association, such as that which would occur if resistance was polygenic. Resistance to *Vibrio* infections has previously been reported to exhibit a polygenic architecture (Yang et al., 2022), and therefore may require a significantly larger sample size to identify a significant association. It is also possible that inspecting other populations of Pacific oysters could identify major effect loci for *Vibrio* survivorship that are not present in the MBP families investigated here. Furthermore, differences in resistance across families could involve other determining factors, such as epigenetic differences impacting immune responses to the bacterial infection. For example, a recent GWAS for

survival to Pacific oyster mortality syndrome indicated that epigenetic factors play a larger role than genetic factors for the syndrome (Gawra et al., 2023). Future studies may benefit from including a larger number of individuals to account for the potential polygenic nature of *Vibrio* resistance mechanisms, but also may need to consider that *Vibrio* resistance as a trait could be best addressed by other selective breeding approaches, including genomic selection (Boudry et al., 2021; Meuwissen et al., 2016), rather than marker-assisted selection, in part because of the challenge in identifying major effect loci.

Conclusion

In this study, we found that the genotype and allelic dosage of the Chr8 OsHV-1 resistance locus did not affect susceptibility to *V. aestuarianus* infection in selectively bred juvenile Pacific oysters. Although this suggests that the Chr8 marker is not genetically correlated to *Vibrio* resistance, our GWAS analysis did not find any loci significantly associated with *Vibrio* survivorship, suggesting that the trait is likely to be controlled by polygenic architecture. We observed significant family differences in *Vibrio* susceptibility, even within the MBP breeding program, which has been selectively bred for at least six generations (de Melo et al., 2016) and is therefore expected to be more genetically homogenous than other outbred oysters. These observations emphasize the potential for further investigation into selective breeding for resistance to *Vibrio aestuarianus*, which may include the use of genomic selection rather than marker-assisted selection based on the lack of major effect loci observed in the present study.

Supplementary Materials

Supplementary tables and figures are provided in the Supplementary Results section.

Additional File S1. Read counts and alignments for all samples retained in the analysis.

Data Availability

Raw sequencing data have been uploaded to SRA under BioProject PRJNA1145972 with BioSamples SAMN43080749 - SAMN43080938.

FigShare RADseq genotypes and supporting files: 10.6084/m9.figshare.26524321

FigShare rhAmp assay raw data plate files: 10.6084/m9.figshare.26515438

FigShare Sample and family IDs for mapping families: 10.6084/m9.figshare.28599923

This analysis was supported by the following repositories:

Manuscript code and README: https://github.com/bensutherland/ms_cgig_chr8_vibrio

simple_pop_stats repository: https://github.com/bensutherland/simple_pop_stats

Funding

Funding for this study was provided by the Pacific States Marine Fisheries Commission (PSMFC) project "Preparing for Future Challenges – Threats from Ocean Acidification, *Vibrio coralliilyticus* and OsHV-1 µvar to West Coast Oyster Farmers" (Award No. NA18NMF4720007).

Conflicts of Interest

BJGS is affiliated with Sutherland Bioinformatics. The author has no competing financial interests to declare. The other authors declare no conflicts of interest

Acknowledgements

The authors wish to thank the members of the Centre for Shellfish Research (Vancouver Island University) for laboratory assistance. The authors acknowledge Antonia Barela, Gary Fleener, and William Schoeneck for their assistance in the hatchery for the production of juvenile oysters. The authors are grateful to the Deep Bay Marine Field Station staff for using their hatchery facility to store and acclimate juvenile oysters. The authors are also thankful to Brian Boyle at IBIS for ddRADseq library preparations and sequencing. We thank the Editor and three anonymous reviewers for comments on an earlier version of this manuscript.

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TABLES AND FIGURES

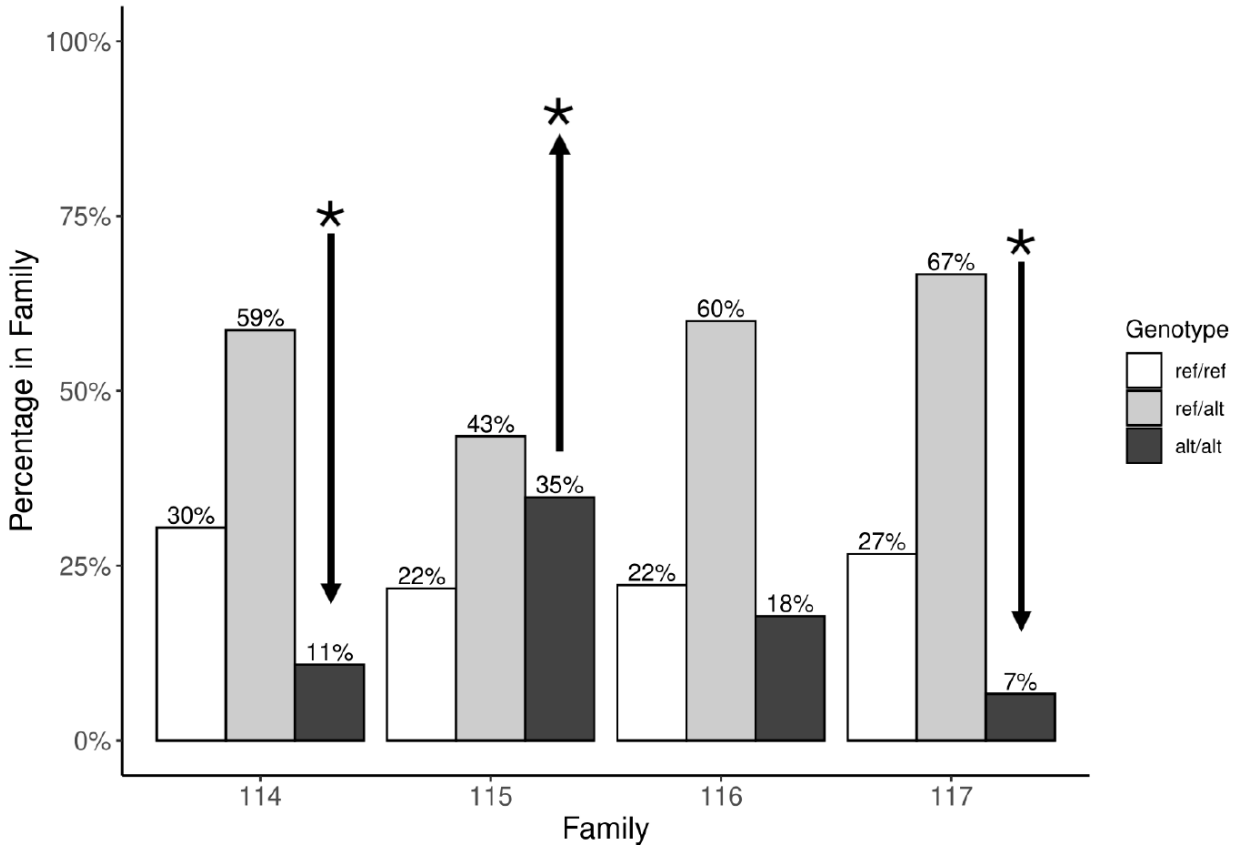

Figure 1. Baseline genotype percentages of OsHV-1 resistance markers in selectively bred mapping families (MBP program F114-F117) before the *Vibrio* exposure trial. **The** genotype is denoted by colour. Columns with arrows indicate a significant difference ($p < 0.05$) in genotype proportions between families. Significance was tested using a Fisher's exact test. Significant differences were observed in the number of homozygous alternate genotypes for F115 compared with F114 and F117 (χ^2=15.183, p-value = 0.019). No significant differences were observed in the number of heterozygotes or homozygous refs across all families.

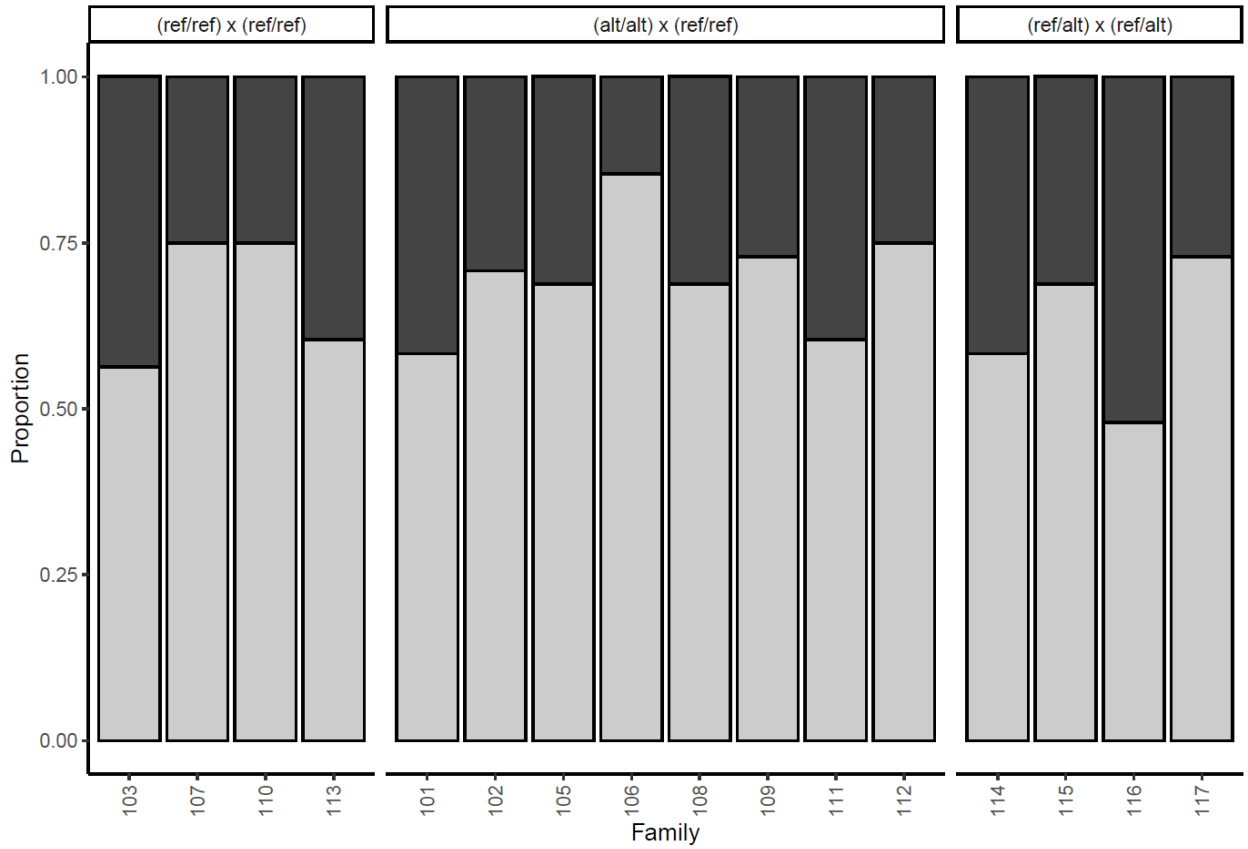

Figure 2. Proportions of oysters that died (light grey) or survived (dark grey) in each family from the *V. aestuarianus* disease challenge. Families are grouped by parental genotype at the OsHV-1 resistance marker, as indicated above the plot. The unexposed control group exhibited 100% survival.

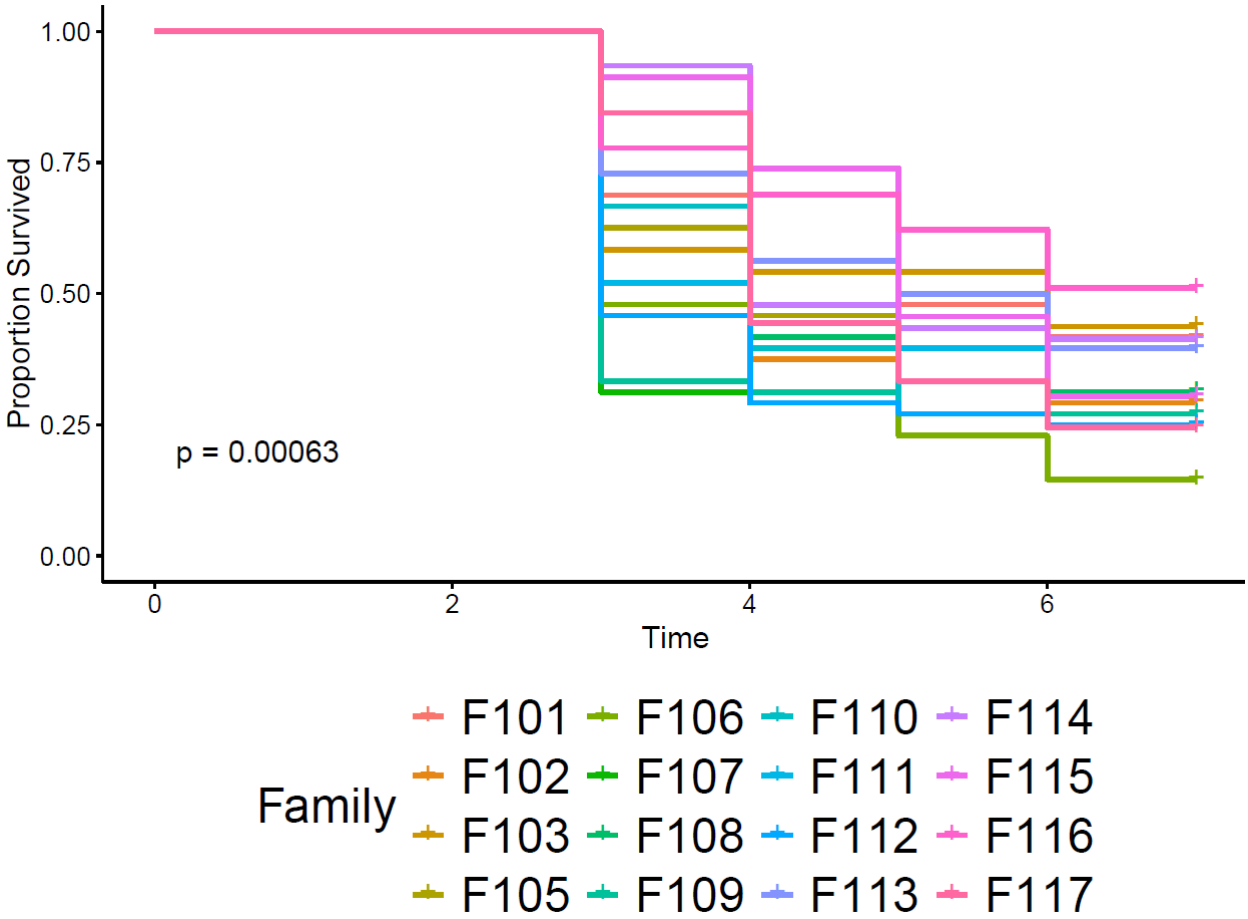

Figure 3. Kaplan-Meier survival curves for Pacific oyster families after exposure to *V. aestuarianus*. Family is denoted by colour. No mortality was observed on days 1 or 2. A log-rank test was conducted to determine whether there were significant differences in the survival distributions for the family crosses. The survival distributions for different family crosses were statistically significant, χ^2=39.070, p<0.001. Pairwise log-rank comparisons were conducted to determine families that had different survival distributions. A Bonferroni correction was made with statistical significance accepted at the p < 0.003 level. Family genotype groupings for the Chr8 marker are given in Figure 2.

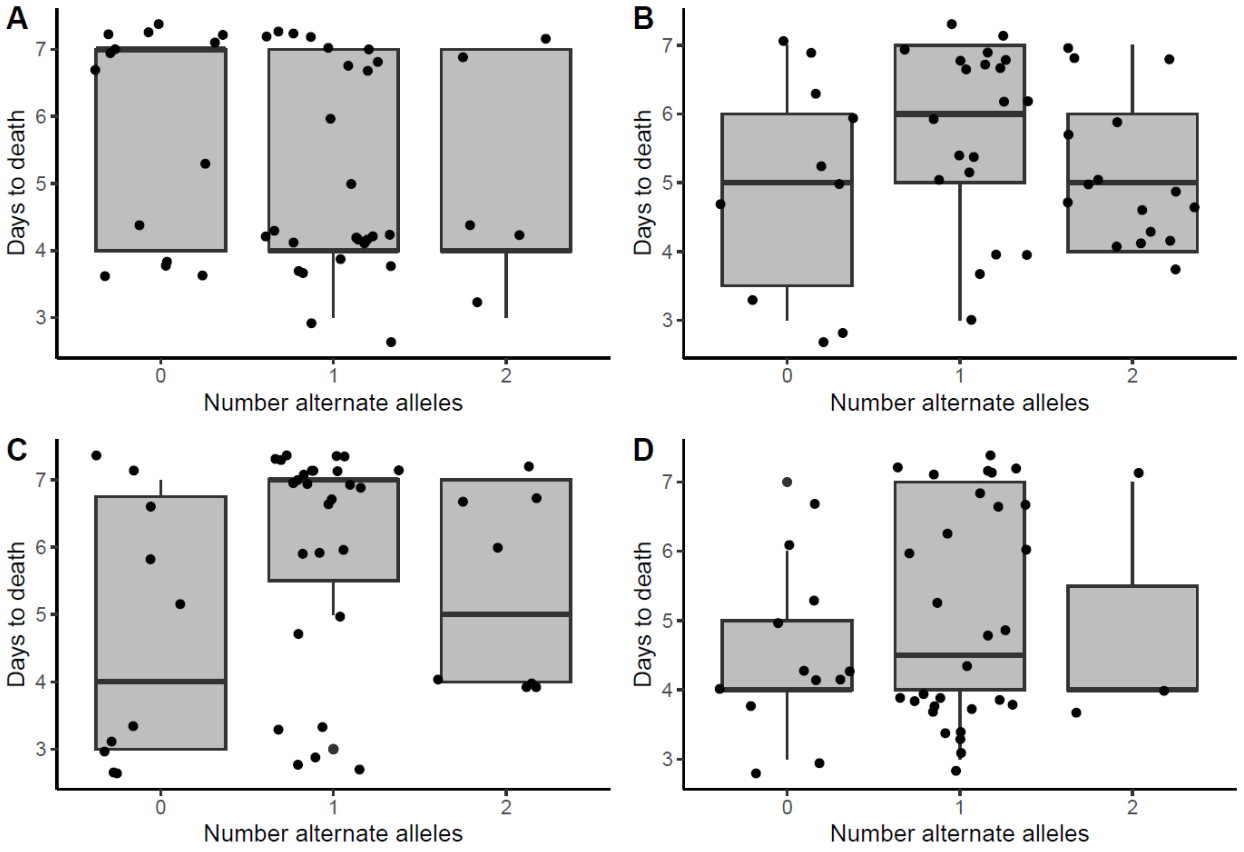

Figure 4. Effects of allelic dosage on mortality (quantified as day of death) to *V. aestuarianus* in oyster mapping families with the OsHV-1 resistance marker (A = F114, B = F115, C = F116, D = F117). Statistical analysis was conducted using a chi-squared test of homogeneity and a Fisher's exact test. No significant association was found between OsHV-1 resistance marker allelic dosage and mortality represented by day of death (p>0.05) (F114 χ^2=5.370, p>0.05; F115 χ^2=14.172, p>0.05; F116 χ^2=11.280, p>0.05; F117 χ^2=4.429, p>0.05).

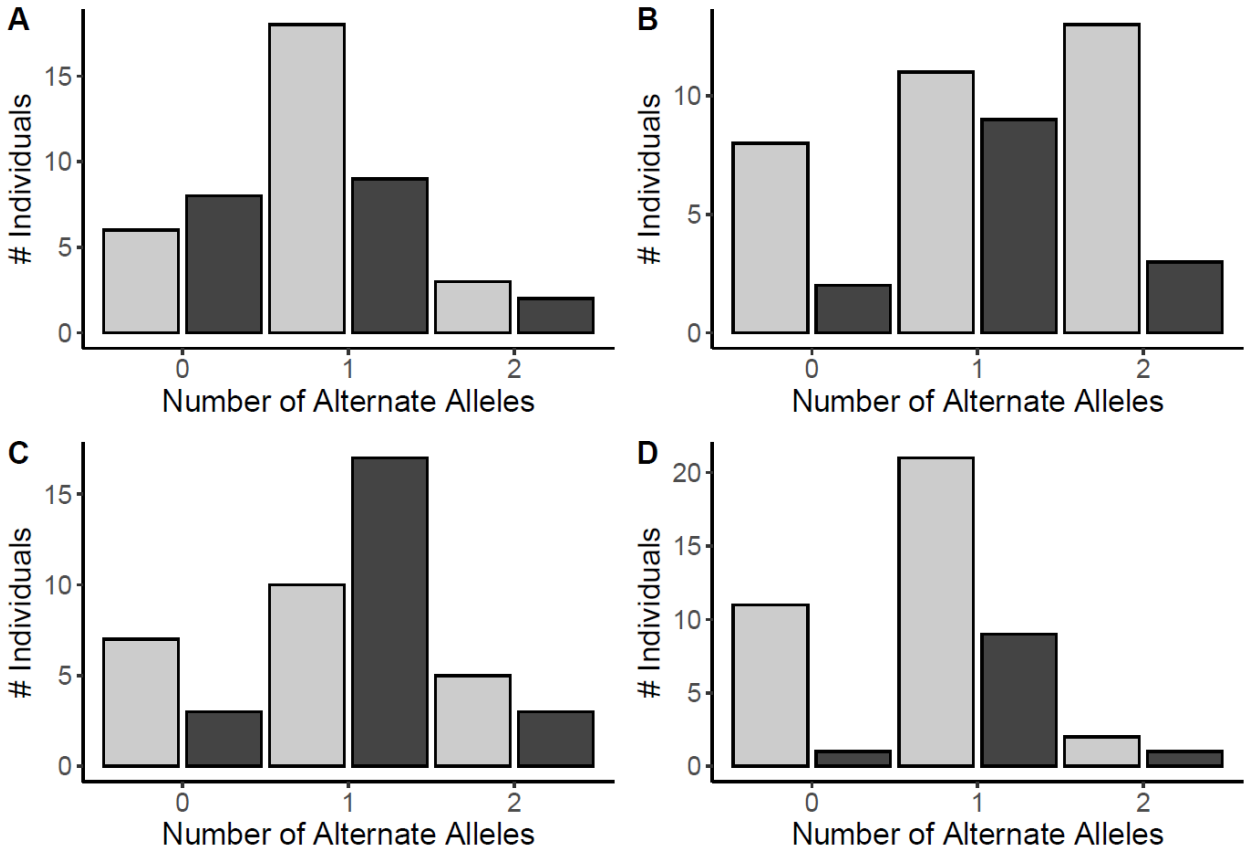

Figure 5. Effects of allelic dosage (i.e., number of alternate alleles) on mortality to *Vibrio* shown as the number of deaths (light grey) compared with the number of survivors (dark grey) for the four families (panel A = F114; B = F115; C = F116; D = F117). Statistical analysis was conducted using a chi-squared test of homogeneity and a Fisher's exact test. No significant association was found between genotype and mortality represented by day of death (p-value >0.05 for all values).

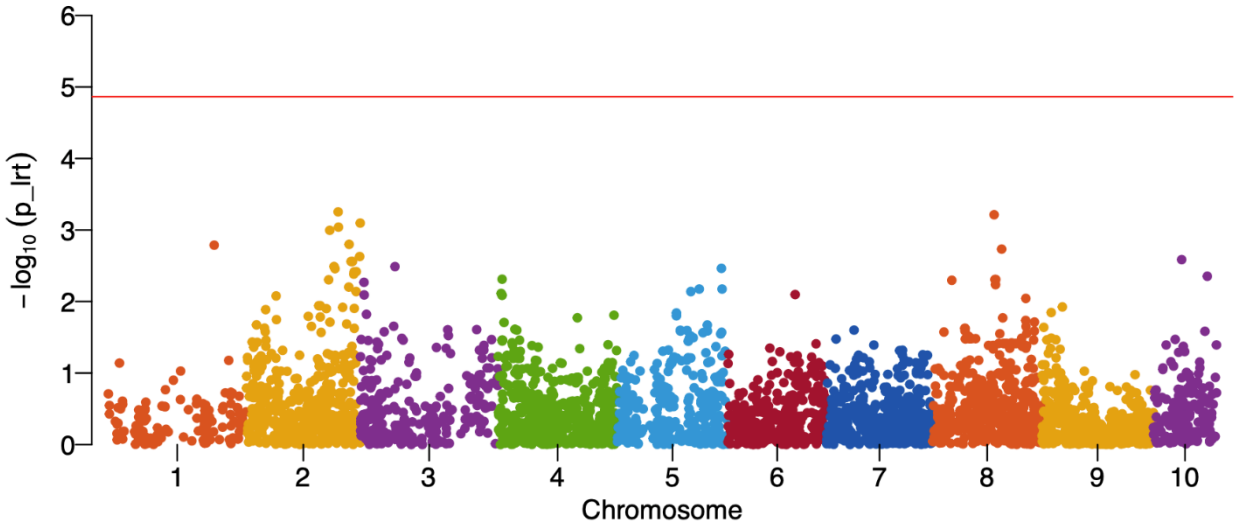

Figure 6. Manhattan plot showing the results of the genome-wide association study (GWAS) for survivorship from the *Vibrio* exposure in Pacific oysters (F114-F117) with the OsHV-1 resistance marker using the phenotype mortality assessed as dichotomous function (died or survived; no imputation applied). The red horizontal line represents the genome-wide Bonferroni-corrected p-value significance threshold of 0.05. No significant associations were observed.