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Infection with *Vibrio aestuarianus* limits the utility of increasing resistance of Pacific oyster *Crassostrea (Magallana) gigas* against OsHV-1 μ Var

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ABSTRACT:

Infection with the oyster herpesvirus type 1 microvariant (OsHV-1 μ Var) has caused mass mortalities of Pacific oyster larvae and spat in multiple countries. Selective breeding to enhance resilience against that virus had been shown as a promising defence strategy. Mass spat mortalities associated with OsHV-1 μ Var affected Pacific oyster farms in Ría de Arousa (Galicia, NW Spain), which led us to explore the potential utility of selective breeding to increase cultured oyster survival. Thus, adult oysters that had survived through culture in that area, heavily affected by OsHV-1 μ Var, and oysters collected from a naturalised oyster bed that had never been affected, were used as broodstocks in hatchery facilities to produce spat families from each origin. Spat families derived from each stock were transferred into a culture raft in Ría de Arousa; survival and occurrence of OsHV-1 μ Var were monitored through cultivation. Spat mortality associated with OsHV-1 μ Var was higher in the families deriving from the naïve stock. Adult oyster mortality was detected close to the end of growing-out, which was not associated with OsHV-1 μ Var but putatively caused by *Vibrio aestuarianus* infection. Adult mortality was higher in the families with the highest *V. aestuarianus* loads; notably, the oyster families with the lowest spat mortality showed the highest adult mortality. Therefore, a potential increase of spat survival in Ría de Arousa through selective breeding to enhance oyster resilience against OsHV-1 μ Var could be counteracted by high adult mortality associated with *V. aestuarianus* infection.

KEY WORDS:

Pacific oyster mortality syndrome · Ostreid herpesvirus · *Malacoherpesviridae* · *Vibrio aestuarianus* · *Crassostrea gigas* · Selective breeding · Aquaculture

1. INTRODUCTION

Summer mass mortalities of Pacific oyster *Crassostrea (Magallana) gigas* spat recurrent since 2008 in France and later in other European countries were associated with the ostreid herpesvirus type 1 micro variant (OsHV-1 μ Var) (Segarra et al. 2010, EFSA AHAW 2015). This phenomenon, called Pacific oyster mortality syndrome, affected Pacific oyster production worldwide (Delmotte et al. 2020, Whittington et al. 2024). Further studies on the disease processes leading to spat mass mortality showed that infection with OsHV-1 μ Var is a first crucial step that diminishes spat immune competence, which is followed by colonization by opportunistic bacterial pathogens resulting in oyster death (de Lorgeril et al. 2018, Petton et al. 2021, Richard et al. 2021, Clerissi et al. 2023, Oyanedel et al. 2023). Selective breeding to increase resistance against OsHV-1 μ Var was shown as a promising strategy to overcome oyster spat mass mortalities (Dégremont et al. 2015a,b,c). However, in addition to spat mass mortalities, the European oyster industry was later struck by summer mortalities of adult Pacific oysters associated with the bacterium *Vibrio aestuarianus* (Barbosa Solomieu et al. 2015, Travers et al. 2017, Lupo et al. 2020, Mesnil et al. 2023). Thus, fighting against Pacific oyster mortality syndrome through selective breeding programmes was revealed as a complex issue, because oysters that had been selected for resistance against OsHV-1 μ Var remain highly susceptible to *V. aestuarianus*, suffering even higher mortality than non-selected oysters (Azéma et al. 2015). Therefore, selective breeding programmes must address dual resistance, against both pathogens, to be efficient in areas affected by these 2 pathogens (Dégremont et al. 2020, 2021).

The Pacific oyster was introduced into Galicia, Spain, for aquaculture in the early 1990s, as a way to mitigate declined production of the native European flat oyster *Ostrea edulis* (Gómez Gesteira et al. 2017). The performance of the Pacific oyster in suspended culture from rafts in Galician estuaries, with quick growth and high survival (Iglesias et al. 2005) and lack of serious pathological threats (Iglesias et al. 2012), facilitated the acceptance and good expectations among Galician oyster farmers. Thus, Pacific oyster culture from rafts spread in Ría de Arousa and Ría de Vigo (SE Galicia), based on growing-out spat imported from France up to market size, while intertidal culture using plastic-net bags laid on metallic frames above the bottom was attempted on various beaches along the Galician coast (Iglesias et al. 2005, Gómez Gesteira et al. 2017). Additionally, naturalised Pacific oyster beds spread in some northern estuaries of Galicia deriving from unintentional introductions of Pacific oysters mixed with batches of *O. edulis* imported from France in the 1980s for growing out in Galicia and/or attempts for intertidal cultivation of Pacific oysters in the 1990s (Iglesias & Gómez Gesteira 2017, Des et al. 2022). The production of Pacific oyster culture in Galicia progressively increased and peaked in 2007 (annual production of 1015 t). However, it abruptly declined in 2008, very likely due to its complete dependence on spat supply from France and, since then, the production has not recovered (Gómez Gesteira et al. 2017). Field experiments performed in the period 2012–2015 to explore whether OsHV-1 μ Var could be hampering Pacific oyster production in Galicia showed that oyster spat suffered mass mortality associated with OsHV-1 μ Var infection in 2 raft culture areas of the Ría de Arousa (Camados D and Grove A; Fig. 1), where Galician oyster culture was mostly carried out, while that virus was not detected in Pacific oysters cultured in the culture raft area Redondela A (Fig. 1) in the Ría de Vigo, where their survival through culture from spat to market size was high (Villalba et al. 2017). Furthermore, the virus was not detected in the naturalised Pacific oyster beds occurring in the northern Galician estuaries, namely in the Ría de Viveiro and Ría do Barqueiro (Villalba et al. 2017). Thus, searching for ways to mitigate Pacific oyster mass mortalities in Galicia became peremptory. Unfortunately, trials to increase Pacific oyster survival in virus affected culture areas by concentrating the first stages of oyster growing-out (when the susceptibility is the highest) in the annual period with lower temperature (when the viral infection is arrested; Petton et al. 2013) were not satisfactory (Villalba & Cao 2017, Villalba et al. 2017).

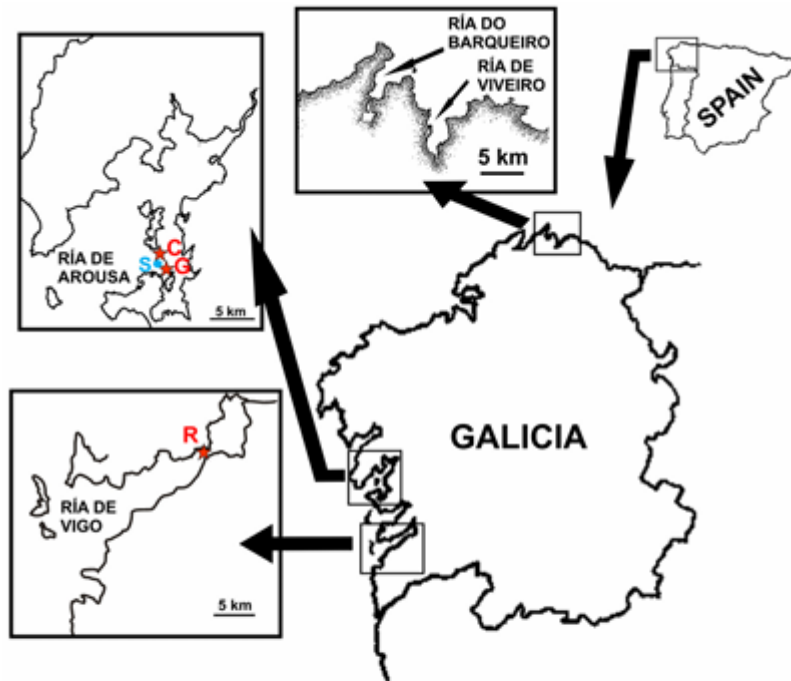


Fig. 1. Galicia (NW Spain), showing the location of the northern estuaries Ría do Barqueiro and Ría de Viveiro and the southwestern estuaries Ría de Arousa and Ría de Vigo. The locations of the areas of raft culture 'Cambados D' (C) and 'Grove A' (G) in the Ría de Arousa and 'Redondela A' (R) in the Ría de Vigo are shown with red stars. The location of the marine station (S) where seawater temperature was recorded is shown with a blue circle

Considering the promising results of selective breeding programmes performed in France to increase the resistance of Pacific oysters against OsHV-1 μ Var (Dégremont et al. 2015a,b,c), the goal of this study was to explore the utility of selective breeding to increase resistance against OsHV-1 μ Var for the Galician farming industry. A field experiment was conducted to compare oyster spat families deriving from survivors to exposure to OsHV-1 μ Var and families deriving from a naïve population, in a OsHV-1 μ Var affected raft culture area of the Ría de Arousa. Oyster families were monitored for 12 mo (from 3 to 15 mo of age) for survival, growth and disease. Specifically, oysters were monitored for OsHV-1 μ Var and *V. aestuarianus*. Here we report the results of that field experiment.

2. MATERIALS AND METHODS

2.1. Production of Pacific oyster seed in hatchery facilities

Adult Pacific oysters were collected from 2 sources in May 2016 and taken to the hatchery facilities of the Marine Science Station of Toralla (ECIMAT, Centre of Marine Research, University of Vigo) to be used as broodstock. The first source was a naturalised Pacific oyster bed located in the Ría do Barqueiro (43°44.7'N, 7°40.4'W; Fig. 1). There were no previous records of abnormal oyster mortalities in that bed, and all previous analyses to diagnose OsHV-1 μ Var performed since 2012 by conventional PCR (cPCR) on Pacific oyster spat samples taken from that bed had been negative (Villalba et al. 2017); that Pacific oyster bed was considered naïve with regard to infection with OsHV-1 μ Var. The second source of Pacific oysters consisted of survivors of a batch of oysters that had been produced in a local commercial hatchery (Re magro, SA), descending from broodstock collected from the same naturalised Pacific oyster bed. The spat produced in the hatchery was transferred to a raft in

the culture area of Cambados D (42°31.0'N, 8°51.1'W; Fig. 1) in December 2014 and suffered high mortality in May 2015 associated with a high percentage of PCR+ for OsHV-1 μ Var (Villalba et al. 2017); the survivors remaining in that raft in May 2016 were used to select the second source broodstock. Various full sib families of larvae from each stock were produced by mixing male gametes from an oyster with female gametes of another after stripping their gonads, in late May 2016. Each larval family was reared separately following conventional procedures for hatchery oyster culture (Cerviño Otero et al. 2017). Eventually, 3 larval families deriving from survivors of the raft culture in the Ría de Arousa (stock A) and 6 larval families deriving from the naturalised bed in the Ría do Barqueiro (stock B) successfully reached the spat stage and were used in the next step.

2.2. Growing-out spat families in a culture area affected by OsHV-1 μ Var

Three spat families of stock A (families A1–A3) and 6 of stock B (families B1–B6), with 2000 individuals per family, were transferred from the hatchery facilities to a raft located in the culture area 'Cambados D' (Fig. 1) on 1 September 2016, an area known to be affected by OsHV-1 μ Var (Villalba et al. 2017). The mean size of the spat of each family at that time is provided in Table 1. The oyster spat culture procedure was similar to that described by da Silva et al. (2005). The spat was divided among lidded, perforated (1 cm mesh) receptacles (250 individuals per receptacle; Fig. 2A). Each receptacle had a label identifying the spat family. The receptacles were put into perforated (2 cm mesh), circular, plastic baskets (4 receptacles per basket) (Fig. 2B). The baskets were strung in stacks with ropes and hung from the raft (Fig. 2C). The stacks were raised on board approximately monthly, the lidded containers and trays were cleaned (or replaced), and all live and dead oysters were counted to estimate monthly (30 d) mortality rates (M30) and cumulative mortalities; dead individuals were removed. Samples for diagnosis were taken occasionally, as explained in Section 2.3. Furthermore, the length of 20 or 30 individuals per family was measured on board with calipers when no oyster samples were brought to the laboratory. Growing-out (and hence monitoring) finished on 6 September 2017; however, monitoring of 3 spat families (B2, B3 and B6) finished earlier (on 13 December 2016) due to the very high mortality that they suffered. When oysters attained sufficient size to be retained in the circular baskets, the lidded receptacles were no longer used.

Table 1. Mean (\pm SD) values of length and total weight of the oyster spat of each family at the transference from the hatchery to the raft for growing out. Sample size was 50 individuals per family

Family	Length (mm)	Weight (g)
Survivors from Ría de Arousa		
A1	15.8 \pm 1.75	0.5 \pm 0.14
A2	9.2 \pm 0.97	0.1 \pm 0.02
A3	13.6 \pm 2.34	0.3 \pm 0.09
Oysters from Ría do Barqueiro		
B1	17.3 \pm 2.05	0.4 \pm 0.08
B2	13.8 \pm 1.69	0.3 \pm 0.10
B3	17.0 \pm 2.06	0.3 \pm 0.12
B4	19.2 \pm 2.35	0.3 \pm 0.08
B5	16.2 \pm 2.19	0.3 \pm 0.09
B6	14.2 \pm 1.37	0.2 \pm 0.06

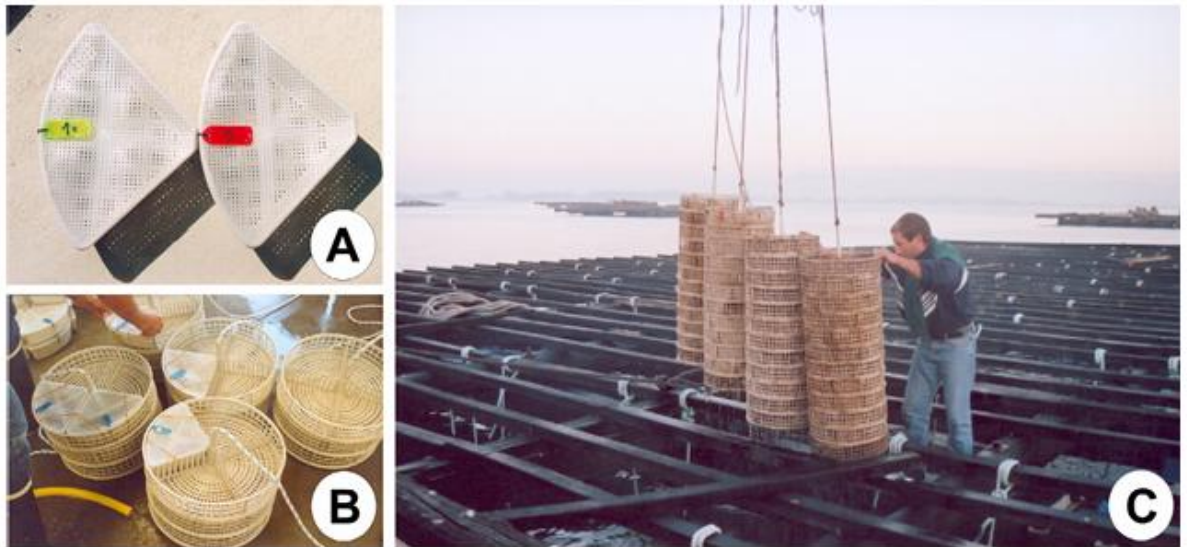


Fig. 2. Lidded receptacles and circular baskets used to grow out Pacific oysters in the study. (A) Lidded, perforated receptacles for small spat. (B) Piled circular baskets, some of which contain lidded receptacles. (C) Four stacks of strung circular baskets in the process of being deployed from a raft

2.3. Sampling for diagnosis

A sampling plan had been designed expecting that high spat mortality associated with OsHV-1 μ Var infection would be concentrated in the first months of the growing-out period and no abnormal mortality would occur in the following months, according to previous experiments (Villalba et al. 2017). Thus, samples for diagnosis of OsHV-1 μ Var by PCR (at least 10 individuals per family) were taken just before transferring the spat from the hatchery to the raft (1 September 2016) and then from the raft (11–15 individuals per family) on 31 October 2016. Unexpectedly, abnormal mortality was detected in family B5 on 3 July 2017, and in family A3 on 3 August 2017. A first reaction involved collecting 30 oysters from those families on those dates, respectively, which were processed for diagnosis of OsHV-1 μ Var by PCR and for histopathological analysis. The possibility of involvement of *Vibrio aestuarianus* in those high-mortality events was further assessed and, at the end of growing-out, on 6 September 2017, 30 oysters from each ‘surviving’ family were processed for the diagnosis of OsHV-1 μ Var by PCR and *V. aestuarianus* by qPCR and for histopathological analysis. The length and the total weight of every oyster processed in the laboratory were measured before shucking. A summary of the sampling events for the different purposes is shown in Fig. 3.

2.4. Diagnosis of OsHV-1 μ Var by PCR

After shucking oysters, whole soft tissues in the case of oyster spat (samples taken in 2016) and gills, digestive gland and gonad pieces in the case of larger oysters (samples taken in 2017) were preserved in dimethyl sulphoxide (DMSO) storage buffer (25 mM EDTA, 20%v/v DMSO and saturated NaCl) for DNA extraction and PCR analysis (Moss et al. 2007). Around 20–25 mg of the preserved soft tissues were homogenised and extractions of DNA were performed employing the QIAamp[®] DNA Mini Kit (Qiagen), following the manufacturer’s protocol. DNA quality and quantity were checked in a Nanodrop[®] ND-1000 spectrophotometer (Nanodrop Technologies). The PCR assay for OsHV-1 μ Var diagnosis was based on the procedure described by Commission Regulation (EU) No 175/2010 (European Commission 2010), with the primer pair CF (5’-CCC CGG GGA AAA AGT ATA AA) and CR (5’-GTG ATG GCT TTG GTC AAG GT) included in that regulation. PCR reactions were performed in a total

The same oysters used for histological analysis were used for diagnosis of *V. aestuarianus* by qPCR. Gills, digestive gland and gonad pieces of each oyster were preserved in DMSO storage buffer. The preserved soft tissues (25 mg) were homogenised and DNA extractions were performed with the QIAamp® tissue Mini Kit (Qiagen), following the manufacturer's protocol. DNA concentrations were measured using a spectrophotometer (Nanodrop, Thermo Fisher) before storage at -20°C . Quantification of *V. aestuarianus* DNA was carried out following the qPCR protocol described by Saulnier et al. (2009) using a genomic standard prepared from *V. aestuarianus* strain 02/041 (Garnier et al. 2008). Amplification reactions were performed with Mx3000 and Mx3005 Thermocyclers (Agilent), in triplicate, in a total volume of 20 μl . Each well contained 5 μl of genomic DNA (adjusted at 5 ng μl^{-1}), 10 μl of Brilliant III Ultra-Fast Master Mix (Agilent), 0.06 μl of each primer at 100 μM (DNAj-F: 5'-GTA TGA AAT TTT AAC TGA CCC ACA A; DNAj-R: 5'-CAA TTT CTT TCG AAC AAC CAC), 0.04 μl of DNAj probe 100 μM (5'-TGG TAG CGC AGA CTT CGG CGA C, dual-labelled with a 5'-reporter dye Texas Red and a downstream 3'-quencher dye BHQ-2, produced by Eurogentec) and 4.84 μl of distilled water. The resulting final concentration of each primer was 0.3 μM and that of the probe was 0.2 μM . Real-time PCR cycling conditions were as follows: 3 min at 95°C , followed by 40 cycles of amplification at 95°C for 10 s and 60°C for 20 s.

2.7. Seawater temperature data

No device was set to record seawater temperature at the trays hung from the culture raft. However, considering the relevant influence of seawater temperature on the OsHV-1 μVar infection processes (Petton et al. 2013, Renault et al. 2014, Martenot et al. 2015, de Kantzow et al. 2016), values of seawater temperature at 4 m depth (the depth at which the oyster trays hung from the raft) were taken from the records of Stn A7 ($42^{\circ}30.50'\text{N}$, $8^{\circ}51.90'\text{W}$; the closest station to the oyster culture raft) of the oceanographic monitoring net of the Instituto Tecnológico para o Control do Meio Mariño de Galicia (INTECMAR), which were accessible through www.intecmar.gal/Ctd/Default.aspx. The distance between the raft where oyster growing-out took place and Stn A7 was ca. 300 m.

2.8. Statistics

As explained in Section 3, we detected 2 periods with high oyster mortality during the study; the first affected oyster spat and the second affected adults. The correlation between the percentage of PCR-positive cases on 31 October 2016 sampling and the cumulative mortality of each family recorded on 13 December 2016 (last sampling in the phase of high spat mortality) was evaluated by calculating Spearman's rank correlation coefficient (r_s); the strength of the correlation was interpreted following the criteria proposed by Fowler et al. (1998). Considering that sampling on 13 December was the last sampling in which significant oyster spat mortality was detected, differences in cumulative mortality at that sampling time between family pairs were assessed with a χ^2 test. Regarding the period of high adult oyster mortality, from 30 May to 6 September 2017, differences in cumulative mortality corresponding to this period between family pairs were also assessed with a χ^2 test. Additionally, differences in survival between the 2 oyster stocks in the 2 periods of high mortality were tested using a non-parametric distribution analysis with Kaplan-Meier estimations; the resulting survival curves for each stock were compared with log-rank and Wilcoxon tests. For the analysis of the high spat mortality, survival data were right censored on 16 December 2016, while for the analysis of high adult oyster mortality survival, data were left-censored on 26 May 2017. All statistical analyses were performed using MINITAB 18 software. Results were considered significant at $p \leq 0.05$.

3. RESULTS

3.1. Performance of oyster families through growing-out

Grow-out extended from 1 September 2016 to 6 September 2017; the mean size of the surviving oysters exceeded 70 mm in length and 40 g in total weight at the end of that period (Fig. 4). Four phases could be distinguished through growing out regarding mortality (Fig. 5): (1) the first phase ran from the beginning to the first mortality assessment (22 September 2016), with no recorded mortality. (2) Very high oyster spat mortality then occurred between that first assessment and the third mortality assessment (13 December 2016), more markedly affecting the families of the naïve stock, particularly families B3, B2 and B6 (Fig. 5), whereas 2 (families A3 and A1) of the 3 families deriving from survivors after field exposure to the virus showed significantly lower cumulative mortality at that time. The mean (\pm SD) value of cumulative mortality of families of the naïve stock on 13 December 2016 was $91.6 \pm 8.18\%$, while that of families deriving from survivors after field exposure to the virus was $70.4 \pm 13.26\%$. The significance of the differences between family pairs in cumulative mortality at the end of this second phase is shown in Fig. 5. Monitoring of the families suffering the highest mortality (B1, B2 and B6) could not be continued after that third mortality assessment because the number of surviving oysters in those families was too low. (3) Next, mortality was negligible for every surviving family from 13 December 2016 to 26 May 2017 (Fig. 5). (4) Finally, in the summer 2017, when the mean length of all surviving families had surpassed 50 mm (adults; Fig. 4), high monthly mortality values were recorded in some families, namely, family B5 on 29 June 2017 ($M_{30} = 41.2\%$), family A3 on 3 August 2017 ($M_{30} = 20.1\%$) and 6 September 2017 ($M_{30} = 46.0\%$) and, to some extent, family A1 on 6 September 2017 ($M_{30} = 10.9\%$), while mortality continued to be negligible in the remaining surviving families (Fig. 5). The significance of the differences between family pairs in the cumulative mortality corresponding to this fourth phase is shown in Fig. 5. Remarkably, families A3 and B5, which were those of each stock with the significantly highest mortality record in the fourth phase, affecting adult oysters, were the families with the significantly lowest mortality record of each stock in the second phase, affecting spat. The statistical comparison of mortality between stocks showed that the stock had a significant effect on the survival of oyster spat during the period of high spat mortality (Table 2). However, stock did not have an effect on oyster survival during the period of high adult oyster mortality (Table 2).

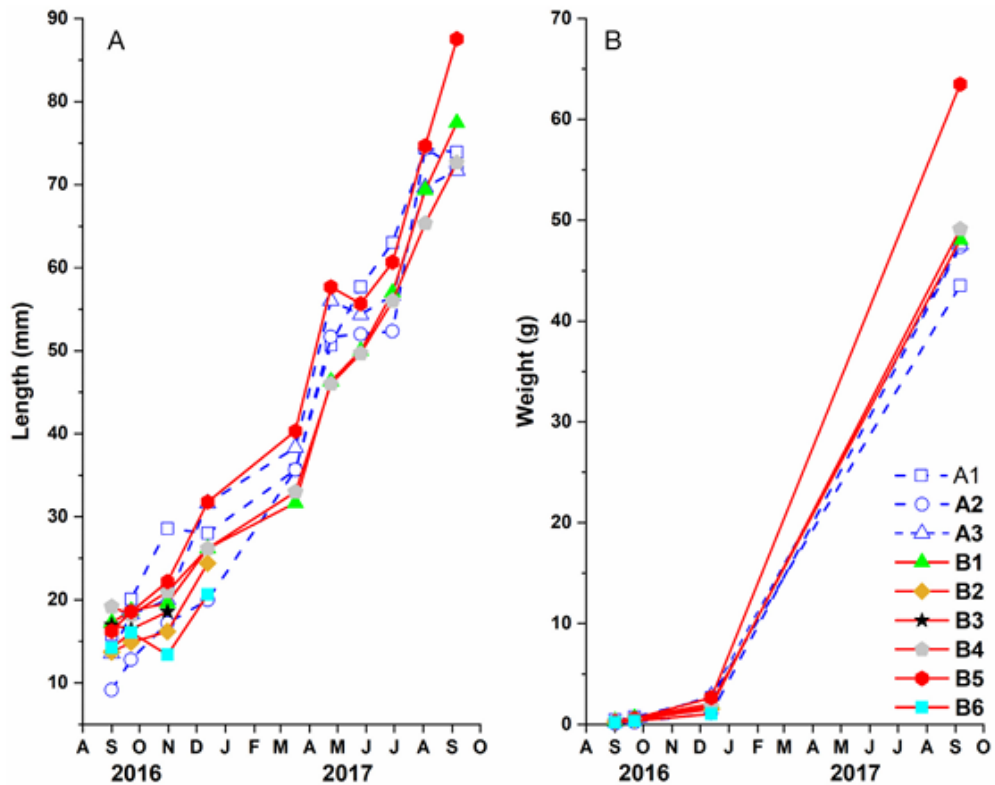


Fig. 4. Temporal variation in the mean values of (A) length and (B) total weight of each Pacific oyster family through the growing-out period. Blue dashed lines correspond to families A1–A3, deriving from survivors that had been cultured in the Ria de Arousa, while red solid lines correspond to families B1–B6, deriving from the naïve stock (Ria do Barqueiro)

3.2. Diagnosis of OsHV-1 μ Var

Table 3 summarises the results of the PCR diagnostic assays for OsHV-1 μ Var. No positive case was detected in any family when assessed at their transference from the hatchery to the culture raft. However, positive cases were detected in every family on 31 October 2016, during the very high spat mortality phase. A significant ($p < 0.001$), albeit modest, correlation ($r_s = 0.59$) was found between the percentage of PCR-positive cases (on 31 October) and the cumulative mortality of each family (on 13 December 2016) in the very high spat mortality phase; in fact, the highest percentages of PCR-positive cases corresponded to the families with the highest cumulative mortality (B2, B3 and B6), which could not be monitored after that phase. In the fourth phase (regarding mortality) of growing-out, 43% PCR positive cases were found in the sample of family B5 taken on 29 June 2017, when high monthly mortality ($M_{30} = 41.2\%$) was recorded. However, no positive cases were detected in the remaining PCR assays from that fourth phase, even in those corresponding to samples from families with moderate or high monthly mortality rates.

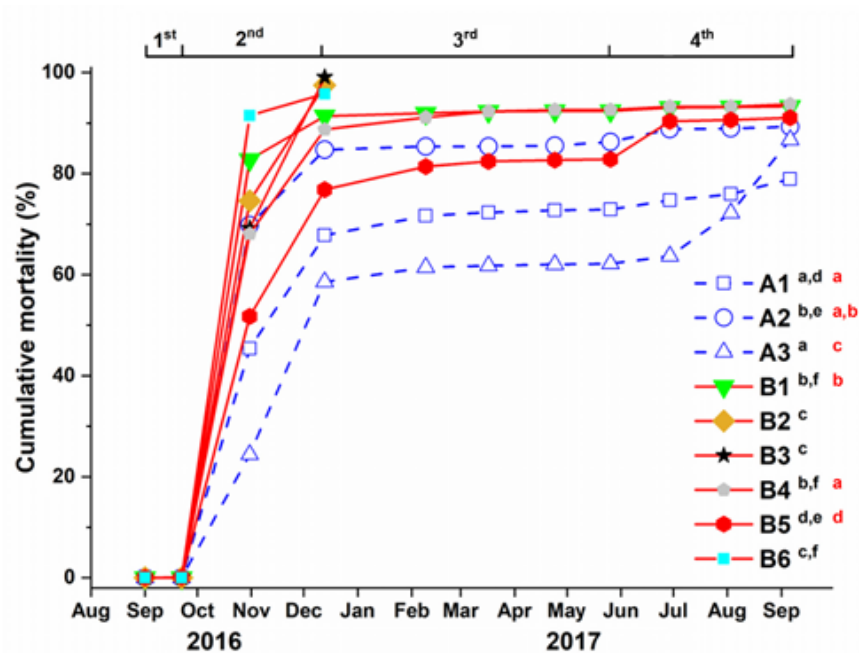


Fig. 5. Temporal variation in the cumulative mortality of each Pacific oyster family through the growing-out period in Ria de Arousa. Blue dashed lines correspond to families A1–A3, deriving from survivors that had been cultured in the Ria de Arousa, while red solid lines correspond to families B1–B6, deriving from the naïve stock (Ria do Barqueiro). The 4 phases of the growing-out period, distinguished by mortality (see Section 3.1) are shown above the graph. Different superscript black letters in the key denote significant differences in cumulative mortality between families sampled on 13 December. Different superscript red letters in the key denote significant differences in cumulative mortality for the period 30 May to 6 September 2017, based on samples collected on 6 September 2017

3.3. Diagnosis of *Vibrio aestuarianus*

The results of the qPCR-assays performed to estimate the load of *V. aestuarianus* in the tissues of live adult oysters collected at the final sampling are shown in Fig. 6. *V. aestuarianus* DNA was detected in every oyster family with variable individual loads (from 102 to 107 bacterial genomic units per 25 ng of total DNA). The highest mean values corresponded to families A3 and A1, which suffered the highest mortality in the period 9 June to 6 September 2017; values were particularly high in family A3. Notably, family A3 was the only family in which *V. aestuarianus* was detected in 100% of analysed oysters.

3.4. Histological analysis

The examination of histological sections of oysters in the samples taken in the last months (Fig. 3) of the growing-out phase showed the occurrence of some symbionts. *Sphenophrya*-like and *Trichodina*-like ciliates were observed on or close to the branchial epithelium and *Ancistrocoma*-like ciliates in the lumina of digestive gland tubules and ducts of some oysters, always with very low abundance (Fig. S1A–C in the Supplement at www.int-res.com/articles/suppl/d161p055_supp.pdf). Intracellular basophilic inclusions resembling intracellular microcolonies of bacteria were rarely observed in the epithelia of the digestive gland (Fig. S1D). A cope pod was detected in the epibranchial chamber of an oyster (Fig. S1E). Additionally, some histological lesions were observed, both in families affected and not affected by abnormal mortality. Namely, we observed smaller to larger areas of lysis in the subepithelial connective tissue of the mantle, connective tissue of the visceral mass and adductor muscle (Fig. S2A–D); detachment of epithelial cells from the mantle (Fig. S2E); atrophy of the epithelium of some digestive gland tubules, thus showing enlarged tubule lumen (Fig. S2F); and

inflammatory reactions including light to heavy haemocytic infiltration of the connective tissue of different organs (Fig. S3A,B), massive migration of haemocytes through digestive epithelia into digestive lumina (Fig. S3C) and occurrence of masses of aggregated haemocytes in the lumina of vessels (Fig. S3D).

Table 2. Results of non-parametric distribution analysis with Kaplan-Meier estimations to compare differences in survival between the 2 oyster stocks (A and B) in 2 periods of high mortality, that of high spat mortality and that of high adult oyster mortality. For each period, estimations of survival probability (and SE) for each stock at each sampling time are provided as well as the results of the comparison of the estimated survival curves of both stocks with log-rank and Wilcoxon tests

Time (d)	Stock A Survival probability	SE	Stock B Survival probability	SE
High spat mortality period (1 Sep–16 Dec 2016)				
60	0.535	0.0091	0.270	0.0057
103	0.297	0.0083	0.085	0.0036
Method	χ^2	df	p-value	
Log-rank	790.517	1	<0.001	
Wilcoxon	736.662	1	<0.001	
Time (d)	Stock A Survival probability	SE	Stock B Survival probability	SE
High adult oyster mortality period (30 May–6 Sep 2017)				
34	0.925	0.0094	0.724	0.0249
69	0.799	0.0143	0.711	0.0253
103	0.573	0.0176	0.680	0.0260
Method	χ^2	df	p-value	
Log-rank	3.22151	1	0.073	
Wilcoxon	0.19622	1	0.658	

3.5. Seawater temperature

Fig. 7 shows the temporal variation in the seawater temperature recorded at 4 m depth close to the culture raft during the oyster growing-out period. Considering as a reference the threshold of 16°C, above which OsHV-1 Var-associated mortalities occur (Renault et al. 2014), the seawater temperature was below that threshold when the oyster spat were hung from the raft (15.46°C was recorded on 5 September 2016) and then oscillated through September below and above that threshold (17.02, 14.76 and 16.05°C recorded on 12, 19 and 26 September, respectively). The temperature mostly remained below that threshold in October but surpassed it in late October 2016 and then dropped below the threshold for the next months. The threshold was exceeded again in mid-May 2017 and mostly remained above 16°C until the end of oyster growing-out, on 6 September 2017 (Fig. 7).

4. DISCUSSION

The experiment reported in this study was designed to explore the utility of selective breeding to increase resistance against OsHV-1 μ Var for the Galician oyster farming industry. As expected,

considering previous experiments (Villalba & Cao 2017, Villalba et al. 2017), oyster spat suffered high mortality during growing-out in Ría de Arousa, which was significantly higher in the oysters of the naïve stock than in those deriving from survivors after field exposure to OsHV-1 μ Var. Interestingly, we distinguished an initial phase of at least 3 wk (1–22 September) with negligible mortality in all families, regardless of stock. That phase was followed by a period (22 September to 13 December) of very high spat mortality. The only sampling to diagnose OsHV-1 μ Var during those 2 phases was performed on 31 October, approximately in the middle of the second phase, i.e. the phase with very high mortality. The significant, modest association of the percentage of spats positive for OsHV-1 μ Var infection of each oyster family at that time with the cumulative mortality of each family at the end of the second phase (13 December) strongly suggested that the high spat mortality was due to infection with OsHV-1 μ Var. Furthermore, the results showed a trend of higher resilience to viral infection in the spat deriving from oysters that survived exposure to the virus compared to the spat deriving from the naïve oyster bed. We do not know whether the spats were infected during the first phase. Nevertheless, according to the temperatures recorded at a station close to the culture raft, we can speculate that infections likely did not progress to a lethal viral load because seawater temperature could have been below 16°C for most of that period. Seawater temperature could have surpassed that threshold at the beginning of the second phase and approximately in the middle of it, which would have enhanced infection progression, causing high spat mortality (Petton et al. 2013, Renault et al. 2014, Martenot et al. 2015). Low seawater temperature, below that threshold, could explain the negligible mortality during the third phase of growing-out. Oyster mortality was recorded again in some families during the fourth (last) phase of growing-out, when they were adults, notably in those families with the lowest mortality records during the spat stage, i.e. the first and second phases of growing-out. The involvement of OsHV-1 μ Var in the high mortalities of the fourth phase could be discarded for most affected families, because every PCR was negative in this period, except in the case of diagnostic assays performed with the June 2017 sample of family B5. The lack of detection of this virus in most samples from the fourth phase, even though seawater temperature was above 16°C, is in accordance with the low susceptibility of adult oysters to this virus (Peeler et al. 2012, Petton et al. 2015). Additionally, most oysters susceptible to the virus in each family would have died during the spat stage. Seawater temperature values higher than 16°C as the threshold for outbreaks of Pacific oyster mortality syndrome have been proposed in other areas, such as in Australia (Paul-Pont et al. 2014), but the differences in threshold temperature between Europe and Australia could be due to the differences in the viral genotype reported between the 2 geographic areas (Trancart et al. 2023).

Table 3. Percentage of PCR-positive cases for OsHV-1 μ Var in each family (see Table 1) at different sampling times. The number of analysed individuals is shown in brackets. NA: not applicable

Date (d/mo/yr)	Ría de Arousa			Ria do Barqueiro					
	A1	A2	A3	B1	B2	B3	B4	B5	B6
01/09/2016	0 (10)	0 (10)	0 (10)	0 (10)	0 (10)	0 (10)	0 (10)	0 (10)	0 (10)
31/10/2016	67 (15)	47 (15)	87 (15)	83 (12)	87	100 (11)	80 (15)	60 (15)	93 (15)
29/06/2017	NA	NA	NA	NA	NA	NA	NA	43 (30)	NA
03/08/2017	NA	NA	0 (30)	NA	NA	NA	NA	NA	NA
06/09/2017	0 (30)	0 (30)	0 (30)	0 (30)	NA	NA	0 (30)	0 (30)	NA

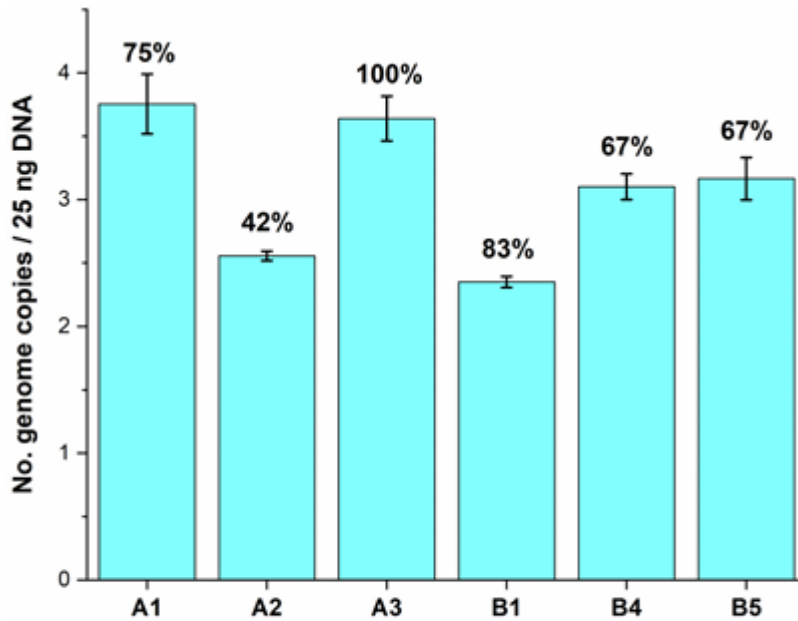


Fig. 6. Mean (\pm SD) values of *Vibrio aestuarianus* load in oysters of each family, expressed as no. of genome copies per 25 ng DNA, at the end of the growing-out period. Values above the bars correspond to the percentage of oysters with PCR-positive results for *V. aestuarianus* in each oyster family

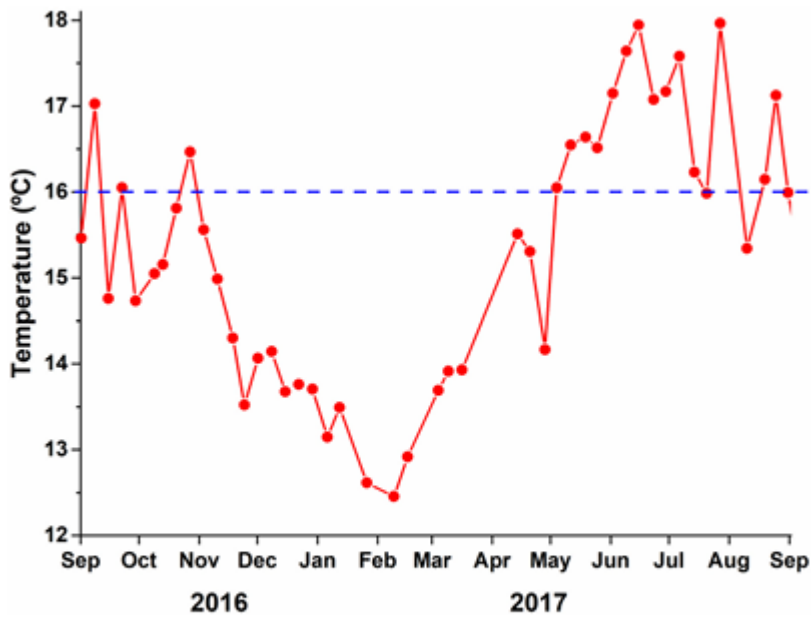


Fig. 7. Temporal variation in seawater temperature at 4 m depth during the oyster growing-out period. Blue dashed line: threshold above which OsHV-1 Var-associated mortalities occur

Regarding the cause of the high adult oyster mortality, moderate to high loads of *Vibrio aestuarianus*, which has been associated with high mortality of adult Pacific oysters in various European countries (Barbosa Solomieu et al. 2015, Travers et al. 2017, Lupu et al. 2020, Coyle et al. 2023, Mesnil et al. 2023), were detected at high prevalence in the all families at the last sampling (on live animals). Globally, high loads are only recorded in moribund or recently deceased animals, and prevalence in infected areas, in the absence of mortality, is quite low (10–25%; Parizadeh et al. 2018).

In this study, the highest mean values and prevalence corresponded to the families suffering the highest mortality in the fourth growing out phase, suggesting that mortality was perhaps still in progress. *V. aestuarianus* is known to induce a chronic-like disease, with a moderate transmission ($R_0 = 2.88$; Lupo et al. 2019), leading to mortality over several months in France (Parizadeh et al. 2018). This high mortality of adult oysters likely associated with *V. aestuarianus* occurred in warm months, consistently with the enhancing effect of high seawater temperature on Pacific oyster mortality caused by *V. aestuarianus* (Lupo et al. 2020).

No significant effect of the stock on adult oyster mortality was detected in our study. Nevertheless, comparisons of mortality rates between oyster groups after very high mortality at the early stage could be biased because high spat mortality leads to selection for the most robust individuals within a cohort and, thus, the individuals remaining are overall less susceptible to all environmental insults. Remarkably, in each stock, the highest mortality in the fourth growing-out phase, which affected adults, corresponded to the family that had shown the lowest mortality at the spat stage. It is known that the immune responses of Pacific oysters against OsHV-1 μ Var are different from those against *V. aestuarianus* (Green et al. 2016) and, thus, the genetic bases of resistance against each of these pathogens are also different (Azéma et al. 2017). Accordingly, selected oyster lines resistant against OsHV-1 μ Var could be more susceptible to *V. aestuarianus* than non-selected lines (Azéma et al. 2015). An alternative explanation would be that the families suffering the least selection of robust individuals (lowest mortality) at the spat stage would include the highest proportion of susceptible individuals to any stress factors at the adult stage.

None of the organisms detected in oysters by histopathological analysis could be blamed for the high adult oyster mortality. Ciliates, intracellular microcolonies (referred to as prokaryote-like colonies) and gill copepods have been reported in Pacific oysters from Galicia, but none was considered as a serious health problem (Iglesias et al. 2012). The histological lesions detected in our study could have resulted from multiple causes, potentially including infection with *V. aestuarianus*. Among them, lysis of subepithelial connective tissue in the mantle, atrophy of the digestive lumen of digestive gland tissues and less to more severe haemocytic infiltration of connective tissue in different organs are histological lesions that have been reported from Pacific oysters infected with *V. aestuarianus* (Parizadeh et al. 2018, Mandas et al. 2020).

In conclusion, our results showed that increasing oyster resistance against OsHV-1 μ Var by selective breeding would not guarantee high oyster survival through growing-out in Ría de Arousa because high adult oyster mortality putatively associated with *V. aestuarianus* occurred. Instead, selective breeding for dual resistance against both pathogens should be accomplished (Dégremont et al. 2020, 2021).

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