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Enhancing resistance to *Vibrio aestuarianus* in *Crassostrea gigas* by selection

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Abstract :

Ostreid herpesvirus 1 (OsHV-1) and Vibrio aestuarianus are the two main pathogens affecting the production of French oyster (Crassostrea gigas). The absence of genetic correlation between the two diseases is promising for the development of stocks with dual resistance. Using unselected and selected oysters concerning enhanced resistance to OsHV-1 infection, we investigated the first generation of mass selection and the response to selection to increase the resistance to V. aestuarianus for two stocks. For each stock, four groups were produced in June 2013 using either parents unchallenged with the bacteria or counterparts that survived experimental infections by the bacteria. Thus, groups were unselected oysters for both pathogens, selected for either the virus or the bacteria, and dually selected for both pathogens. All groups of each stock were evaluated at the spat and juvenile sizes following experimental infection by V. aestuarianus in May 2014. Regardless of their level of selection for OsHV-1, oysters produced from parents that survived V. aestuarianus showed similar mortalities (47% and 53% for stocks A and B, respectively) during the bacterial challenge than those produced from unchallenged parents (43% and 56%, respectively). Thus, no positive response to selection at the first generation to increase the bacterial resistance was found at the spat and juvenile sizes. At the adult stage and with experimental infection with V. aestuarianus, only stock B showed a positive response to selection for increasing the bacterial resistance with a decrease in mortality of 14% in comparison with unselected oysters. Similar results were observed when oysters were tested for 27 months in the field with the absence of response to selection for stock A, while a 13% decrease in mortality was observed for stock B. For stock B, mortality at endpoint after 27 months in the field reached 89% for the control not selected at all, decreased to 84% for oysters selected for their resistance to the bacteria, was 53% for the oysters selected for their resistance to OsHV-1, and was 32% for oysters selected for dual resistance. Realized heritability estimated at the first generation of mass selection for stock B was higher for oysters selected for dual resistance, ranging from 0.47 to 0.80, than those only selected for V. aestuarianus, ranging from 0.05 to 0.30. Selection for dual resistance in C. gigas could limit the impact of both OsHV-1 and V. aestuarianus on oyster production.

Highlights

► This is the first investigation of mass selection to enhance resistance to *Vibrio aestuarianus* in *Crassostrea gigas.* ► Only one stock showed a positive response to selection. ► Stock B showed a gain of survival of +14% for experimental infection at the adult stage. ► Dual selection for OsHV-1 and the bacteria increased the field survival of *C. gigas* from 11% to 68% for stock B.

Keywords : *Crassostrea gigas*, Realized heritability, *Vibrio aestuarianus*, OsHV-1, Disease resistance

1. Introduction

The French oyster industry has regularly suffered from episodes of massive mortality. The most striking examples are the collapse of the production of the Portuguese oyster (*Crassostrea angulata*) related to an iridovirus (Comps and Duthoit, 1976) and the flat oyster (*Ostrea edulis*) due to *Martelia refringens* and *Bonamia ostreae* (Grizel, 1983). To sustain the French oyster industry, *C. gigas* was introduced in France during the 1970s (Grizel and Héral, 1991). *C. gigas* now represents 98% of the French oyster production (FAO, 2019). Unfortunately, this species is currently threatened by two pathogens that produce high mortality rates. The Ostreid herpesvirus 1 (OsHV-1) has produced mortality rates exceeding 70% in spat and juveniles, since 2008 while market-size adults have suffered similar mortality due to the bacterium *Vibrio aestuarianus* since 2012 (Azéma et al., 2017b). As a direct consequence of these two diseases, the French oyster production has decreased significantly from 111 000 tons in 2007 to 64 000 tons in 2016 (FAO, 2019).

A selective breeding program to enhance disease resistance using the genetic resources available in oyster populations is an attractive option to reduce their impact on oyster production (Dégremont et al., 2015a). Such a strategy is commonly and successfully used in aquaculture species (Gjedrem and Rye, 2018; Hollenbeck and Johnston, 2018; Houston, 2017). The high genetic basis for survival for *C. gigas* at the spat stage, and therefore to OsHV-1, infection has been demonstrated (Dégremont et al., 2010b; Dégremont et al., 2015c). Recently, Azéma et al. (2017b) showed that resistance to *V. aestuarianus* in *C. gigas* had a low to moderate genetic basis at the juvenile and adult stages, and more importantly, the absence of genetic correlation between the resistance to OsHV-1 infection and the resistance to *V. aestuarianus* infection. Although a positive genetic correlation would speed up breeding resistance, dual resistance to both diseases could still be implemented in *C. gigas*.

The response to selection to increase the resistance to OsHV-1 infection has been successfully demonstrated in *C. gigas* under field conditions (Dégremont et al., 2015b), while it remains to be investigated for the resistance to *V. aestuarianus* infection. In this study, we investigated this gap by testing the progenies from survivors of experimental infection by *V. aestuarianus*, which were compared with their counterparts produced from parents not exposed to experimental infection with the bacterium. The response to selection was analyzed under experimental infections, as well as under field conditions where *V. aestuarianus* is naturally present.

2. Materials and methods

2.1. Oysters unselected and selected concerning their resistance to OsHV-1

A mass selection scheme to increase resistance to OsHV-1 infection in *C. gigas* was performed for two stocks (A and B) of wild oysters sampled in 2008 from two sites in the Marennes-Oléron Bay (Charente Maritime, France). For each stock, a base population, G0, was produced in 2009. A sub-sample was kept in our facilities at Ifremer in La Tremblade to avoid disease-related mortality and to produce the control lines of the following generation (G1-AC and G1-BC, stock A Control line and stock B Control line, respectively) (Fig. 1). The other sub-sample of oysters was deployed in the field, where mortality outbreaks caused by OsHV-1 have been routinely observed each year since 2009 (Fig. 1) (Dégremont et al., 2015b). The survivors were spawned to produce the selected lines G1-AS (G1 – stock A, OsHV1-Selected line) and BS (stock B, OsHV1-Selected line) in 2010. The same approach was used in February 2011 and March 2012 to produce G2 and G3, respectively, with the exception that four replicate batches were produced for the selected line from G2. Further

details are given in Dégremont et al. (2015b). The oysters used as parents in this study were the control lines G3-AC and G3-BC, and the best replicate batch of the selected lines G3-AS and G3-BS showing the highest survival, and thus the highest resistance to the infection by OsHV-1 (Fig. 1). The estimated inbreeding rates were 0.08 and 0.08 for the AC and BC, respectively, and 0.09 and 0.11 for AS and BS, respectively according to Dégremont et al. (2015b).

2.2. Oyster brood stocks unselected and selected for their resistance to *V. aestuarianus*

For each line (AC and AS for stock A, and BC and BS for stock B), the oysters used as parents were kept in our controlled facilities to avoid disease-related mortality, to produce one control batch, or the survivors from two primary experimental infections by *V. aestuarianus* conducted in February and in March 2013 were kept to produce two selected batches (Fig. 1). The experimental infections used a by-cohabitation protocol corresponding to sets 1 and 2 of trial 3 described in Azéma et al. (2015). To summarize, 75 juvenile oysters per line (AS, BS, AC, and BC) were placed in contact for 48 h with donors injected with *V. aestuarianus*. Mortality was recorded until day 11 post-infection. All survivors were kept at the Ifremer facilities in La Tremblade until spawning in June 2013. Low mortality (15%) occurred during this period. This mortality was related to the detection of *V. aestuarianus* (100% detection from 6 moribund oysters sampled). The final survival of each line from each experimental infection until spawning is shown in Table 1, as well as the corresponding intensity of selection. To summarize, this study involved two stocks (A and B), either selected (AS and BS) or unselected (AC and BC) for their higher resistance to OsHV-1 infection occurring under field condition. The stocks were either unchallenged (control and selected for resistance to OsHV-1 per stock) or challenged with *V. aestuarianus* under controlled experimental

infection conditions (selected for resistance to the bacteria and selected for dual resistance in duplicate per stock), corresponding to a total of four groups per stock. These groups include a control group (unselected for both pathogens one batch), a group selected against the bacteria (two batches), a group selected against the virus (one batch), and a group selected for dual resistance (two batches) (Fig. 1).

2.3. Production of the four oyster groups

From May 2013 until spawning, all parents were placed in the conditioning room at the Ifremer hatchery in La Tremblade. The survivors of the experimental infection were kept in a separate tank from their counterparts that were not exposed to *V. aestuarianus* to avoid the potential for horizontal transmission of putative causal agents of the mortality. The seawater temperature was ambient temperature, ranging from 15 to 22°C, which was enriched with a cultured phytoplankton diet (*Isochrysis galbana*, *Tetraselmis suecica*, and *Skeletonema costatum*) to favor gametogenesis

Spawning was induced in June 2013. For each of the 12 oyster batches, oysters were placed in a 5-L glass beaker filled alternatively with unheated or heated seawater to 28°C to induce spawning. As soon as the first gametes were released, seawater was maintained at 25°C. Every 15 min, the water was sieved, and fertilized eggs were collected on a 20 µm screen and transferred to a 30-L tank. The empty beaker containing the parents was refilled to allow spawning to continue until the end of the spawning event to maximize the number of breeding individuals for each oyster group (Table 1). After spawning, the parents were returned to their respective tanks to be used for subsequent experimental infections by *V. aestuarianus* as described in trials 3 and 4 in Azéma et al. (2015).

The methodology used for the larvae and spat cultures is described in Dégremont et al. (2005) and Dégremont et al. (2007). All oyster groups were kept in our controlled facilities using ultraviolet (UV)-treated seawater, until their evaluations either under experimental infections by *V. aestuarianus* or OsHV-1, or under field condition where both pathogens have been regularly detected in moribund oysters (Azéma et al., 2017a).

2.4. Trial 1: experimental infection with *V. aestuarianus* at the spat and juvenile sizes

In May 2014, the four control oyster batches (AC, AS, BC, and BS) and the eight selected batches of the first generation of mass selection to increase resistance to *V. aestuarianus* were experimentally challenged with *V. aestuarianus* strain 02/041 for 13 days by-cohabitation with naïve oysters injected with *V. aestuarianus*. The cohabitation protocols were previously described in De Decker and Saulnier (2011) and modified for the genetic evaluations of the resistance to this bacteria in *C. gigas* (Azéma et al., 2015; Azéma et al., 2017b).

Naïve oysters (unselected juvenile oysters weighing 5-10 g) were relaxed in a solution of magnesium chloride ($MgCl_2$, 50 g/L) in a mixture of seawater and distilled water (1:4, v:v) for 4 h. Oysters unable to close their valves were injected with 50 µL of a bacterial suspension into the adductor muscle using a 1 mL micro-syringe equipped with an 18-G needle. The bacterial concentration was evaluated spectrometrically at 600 nm and adjusted to an optical density (OD) = 1 representing 5×10^7 colony forming units per mL. The injected oysters were transferred into 10-L tanks for 24 h. In the second step, a ratio of 10 g of injected oysters (with the shell) per 10 L of seawater was used. The injected oysters were placed in contact with the 12 oyster lines for 48 h using three 120-L tanks filled with filtered and UV-treated seawater and maintained at 21°C with adequate aeration and without added food. The salinity averaged 32‰. In each tank and for each batch, two sizes were tested by sampling 25 small (3 g) and 15 large (12 g) oysters corresponding to the spat and juvenile sizes according to the

local oyster farmers, each being maintained inside a small soft mesh labeled with the name of each group. A control tank used the same protocol but with naïve oysters injected with sterilized artificial seawater and placed with the 12 oyster batches. In each tank, dead oysters were counted and removed on days 5, 9, and 12 post-infection, and six moribund oysters were sampled for the detection and the quantification of OsHV-1 and *V. aestuarianus*.

2.5. Trial 2: experimental infection with OsHV-1 in *C. gigas* at the spat and juvenile sizes

Although the main objective of the study was to investigate the response to selection for the first generation of mass selection to increase the resistance to *V. aestuarianus* in *C. gigas*, we were also interested in evaluating their resistance to OsHV-1. As in trial 1, the same protocol was used, except that the trial occurred in June 2014 and naïve oysters were injected with a viral suspension (concentration 2.8×10^4 OsHV-1 DNA copies per µL).

2.6. Trial 3: experimental infection with *V. aestuarianus* in *C. gigas* at the adult size

As in trial 1, the same protocol was used, except that only one size was tested using 15 oysters per batch in each of the three 120 L tanks. Each oyster was individually labeled with a tag. The mean individual weight was 47 g corresponding to the adult size and the trial was performed in January 2015 and lasted 16 days. Dead oysters were removed daily.

2.7. Field testing

The 12 batches were deployed in our oyster farms at Agnas in Marennes-Oléron Bay (45°52'23"N, 1°10'15"W) in December 2013. For each batch, two bags containing 150 spat each at a mean individual whole weight of 2.4 g were tested until February 2016. Oysters were checked monthly from deployment to May 2014. When moribund oysters were

observed, mortality was recorded twice during the peak of mortality in June 2014, in August 2014, in October 2014, in April 2015, and at the endpoint to establish the cumulative mortality. According to Azéma et al. (2017a) and Dégremont et al. (2019), the mortality during the period from deployment to October 2014 for the oysters tested at the spat/juvenile stage should likely be related to OsHV-1 and, to a lesser extent, *V. aestuarianus*, while the mortality of the test adult oysters from October 2014 to February 2016 should be more likely related to *V. aestuarianus*. During the peak of mortality, 13 moribund oysters were sampled for disease screening. In contrast only one moribund oyster was found in April 2015 and for the other counting dates. The total weight of live oysters was recorded in October 2014, April 2015, and February 2016 to estimate the individual weight of the oysters throughout the field testing. This provided a benchmark of growth to compare with life stages commonly used by the French oyster farmers (spat < 5 g juvenile 20 g < adult). Seawater temperature was recorded every hour using two ThermoTrack probes (Progesplus, Willems, France).

2.8. Disease screening

All moribund oysters collected in both experiments were individually analyzed to detect OsHV-1 and *Vibrio aestuarianus*—two main pathogens involved in *C. gigas* mortality in France since 2008. Total DNA was extracted from mantle and gill tissue fragments using the QIAamp tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, as previously described by Schikorski et al. (2011). The total DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and adjusted to 5 ng/µL.

OsHV-1 DNA was detected and quantified with the SYBR® green real-time PCR protocol described by Pépin et al. (2008) and adapted for use with the DPFor/DPRev primers to target

the OsHV-1 DNA polymerase sequence (open reading frame [ORF] 100; DPF 5' ATT GAT GATGTG GAT AAT CTG TG 3' and DPR 5' GGT AAA TAC CAT TGG TCT TGTTCC 3'; (Pépin, 2013). *V. aestuarianus* DNA was detected and quantified by real-time PCR according to the protocol described by Saulnier et al. (2009). The primers used were: DNAj-F 5' GTATGAAATTTTAACTGACCCACAA3'; DNAj-R 5' CAATTTCTTTCGAACAACCAC 3'; and DNAj probe 5' TGGTAGCGCAGACTTCGGCGAC). The real-time PCR cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 20 s. Negative (DNA-free) controls were included. The results are expressed as viral or bacterial DNA copy number per 25 ng of total DNA.

2.9. Data analyses

2.9.1. Mortality

All statistics were performed using SAS® 9.4 software (SAS Inc., Cary, NC). The mortality at endpoint was analyzed within stock for the trials 1 and 2 by a binomial logistic regression throughout the GLIMMIX procedure using a full model including the fixed factors of selection for each pathogen, size, and their interactions, as well as the random factors tanks and batches nested within the selection. Random factors and interactions that were all not significant were dropped from the subsequent analysis. A GENMOD procedure was then used following this reduced model:

$$\text{logit}(\pi_{ijk}) = \mu + \text{size}_i + \text{V. aestu}_j + \text{OsHV-1}_k + (\text{V. aestu} \times \text{OsHV-1})_{jk}$$

where π_{ijk} is the probability of the mortality at endpoint for oyster of the *i*th size (spat vs juvenile) for the *j*th level of selection for *V. aestuarianus* (selected vs control) at the *k*th level of selection for OsHV-1 (selected vs control), and μ the intercept.

For the experimental infection by *V. aestuarianus* for adult oysters in trial 3, the same model was used but without the size factor.

Finally, the mortality in field condition was analyzed using the same model as trial 3 for three periods (1) from December 2013 to October 2014, (2) from October 2014 to February 2016, corresponding to the spat/juvenile mortality and adult mortality, respectively, and (3) from December 2013 to endpoint corresponding to the cumulative final mortality.

2.9.2. Estimation of the realized heritability for the resistance to *V. aestuarianus*

A first generation of mass selection to increase the resistance to *V. aestuarianus* was applied using the survivors from the experimental infections. The resistance to *V. aestuarianus* has an underlying continuous variable, termed the liability with a threshold beyond which oysters will survive, while the others will die. Such a selection on a threshold trait allowed the estimation of the realized heritability for the resistance to *V. aestuarianus*, assuming the liability was normally distributed (Falconer and Mackay, 1996).

Oyster batches were tested in the field for which environmental conditions or disease pressure could vary spatially and temporally. Thus, there was a possibility that changes in the selected groups may result from systematic changes in environmental conditions, or environmental fluctuations may increase the variability of the trait under selection (Roff, 1997), i.e., that threshold may not be fixed between generations. A control batch for each line AS, AC, BS, and BC was produced to assess the effects of changing the environmental conditions during the course of the experiment (Fig.1). Thus, for each stock, the group selected for resistance to the bacteria was used as control for the control group, while the control of the group selected for dual resistance was the group selected against the virus only (Fig. 1).

Assuming unequal variances of liability between relatives of surviving individuals to that in the population before selection, the realized heritability was estimated according to formulae

18.2 and 18.3 in Falconer and Mackay (1996). The sign of the square root was taken to make t between 0 and 1, and using the Appendix Table A in Falconer and Mackay (1996):

$h^2 = t/r$ with $t = \frac{Xc - Xs\sqrt{\left[1-(Xc^2-Xs^2)(1-(\frac{Xc}{i})\right]}}{i+Xs^2(i-Xc)}$

where the subscripts C and S refer to the control and the selected line respectively, X is the normal deviate of the threshold from the mean, i is the mean deviation of the selected parents (the survivors) from the population mean, and r is the coefficient of relationship which equals one when the relatives are offspring of two parents that survived the mortality outbreak (Falconer and Mackay, 1996). The variance of the heritability was computed from the replicate lines (i.e., h^2 was computed separately for each line and from these, the variance in h^2, as described by (Hill, 1972; Roff, 1997).

The realized heritability was estimated only for selected lines showing a positive response to selection to increase the resistance to *V. aestuarianus*, such as in trial 3 (experimental infection by *V. aestuarianus* at the adult stage) as well as for the field exposure for the three periods from December 2013 to October 2014, October 2014 to February 2016, and December 2013 to endpoint.

3. Results

3.1. Trial 1: experimental infection by *V. aestuarianus* in spat and juvenile *C. gigas*

No mortality occurred in the control tank. In contrast, for stock A and at the spat size, oysters selected only for *V. aestuarianus* had the highest mortality (50%) at the endpoint, while oysters selected only for OsHV-1 had the lowest (27%) (Fig. 2A). All groups had significantly higher mortality (+15% on average) at the juvenile size than at the spat size ($P < 0.01$; Table 2, Fig. 2B). The interaction between the selection for OsHV-1 and the selection

for *V. aestuarianus* was not significant (P = 0.91; Table 2). From both sizes, unselected (control and OsHV-1 selected groups) and selected (*V. aestuarianus* [Va] selected and dual selection groups) oysters for bacterial resistance had similar mean mortality with 43 and 47%, respectively (P = 0.30; Table 2). In contrast, unselected oysters for OsHV-1 resistance (control and Va selected groups) had significantly higher mean mortality (53%) than the 39% for oysters selected for OsHV-1 resistance (OsHV-1 selected and dual selection groups) with the odds ratio (OR) of death = 1.83 (P < 0.01; Table 2).

For stock B, the mortality at the spat size at endpoint ranged from 36% for the oysters selected for dual resistance to 70% for the control group (Fig. 2A). The mortality at the juvenile size ranged from 44% for the oysters only selected for OsHV-1 to 67% for the control group (Fig. 2B). Oysters at the juvenile size had significantly higher mortality (58%) than those at the spat size (50%) with the OR of death = 1.36 (P = 0.04; Table 2). The interaction between selection for OsHV-1 and selection for *V. aestuarianus* was not significant (P = 0.21; Table 2). For both sizes and for OsHV-1 selection, unselected oysters (control and Va selected groups) had significantly higher mean mortality (63%) than selected oysters (OsHV-1 selected and dual selection groups) (45%) (P < 0.01, OR = 2.42; Table 2). In contrast, unselected and selected oysters for bacterial resistance had similar mean mortality with 56 and 53%, respectively (P = 0.17; Table 2).

3.2. Trial 2: experimental infection by OsHV-1 in spat and juvenile *C. gigas*

No mortality occurred in the control tank. In contrast, both stocks had similar mortality patterns at the spat and juvenile sizes (Fig. 3A, B). Oysters at the spat size had similar mean mortality across the four groups (64%) than those at the juvenile size (63%) for stock B (P = 0.84). The 57% mortality rate of oysters at the juvenile size was significantly lower than the

rate of 64% at the spat size for stock A (P = 0.04; Table 2). The interaction between the selection for OsHV-1 and the selection for *V. aestuarianus* was not significant for both stocks (stock A: P = 0.81; stock B: P = 0.22; Table 2). Thus, selected oysters for OsHV-1 (OsHV-1 selected and dual selection groups) had significantly lower mortality (27-32%) than the 94% for unselected oysters (control and Va selected groups) for both stocks (P < 0.01; Table 2). The OR of death in unselected oysters for stock A and B was 39.82 and 31.80 times that of the selected oysters, respectively (Table 2). In contrast, oysters selected for their resistance to *V. aestuarianus* (Va selected and dual selection groups) had similar mortality to the unselected oysters (control and OsHV-1 selected groups), with 60% and 63% for the stock A (P = 0.50), respectively, and 63% and 64% for the stock B (P = 0.68), respectively (Table 2).

3.3. Trial 3: experimental infection by *V. aestuarianus* in adult *C. gigas*

No mortality occurred in the control tank. In contrast, mean mortality among all groups and stocks reached 32% at the endpoint, ranging from 24% for the Va selected group to 36% for the dual selection group for the stock A, and ranging from 29% for the Va selected group to 46% for the control group for stock B (Fig. 4). None of the factors was significant (P > 0.05), except for the bacterial selection factor for stock B (Table 2). The selected oysters of the stock B (Va selected and dual selection groups) had a significant lower mortality (30%) than the 44% of the unselected oysters (Control and OsHV-1 selected groups) (Table 2, Fig. 4).

3.4. Field testing

The seawater temperature at Agnas from December 2013 to February 2016 is provided in Supplementary Fig. 1. The mean cumulative mortality recorded throughout the field study for each group are reported in Supplementary Fig. 2A for stock A and in Supplementary Fig. 2B

for stock B. The mean individual weight increased from 2.4 g at deployment in December 2013 to 41.8 g in October 2014, to reach 51.4 g in April 2015 and 66.2 g at the endpoint in February 2016.

No mortality was observed from deployment to mid-May 2014. A mortality outbreak occurred in June 2014 when seawater reached and remained above 16°C (Supplementary Fig. 1, 2A, 2B).

After the first growing season in October 2014, the mean mortality among the four groups reached 59% and 52% for stock A and B, respectively, ranging from 20% for the dual selected group of the stock B to 80% for the VA group of stock A (Fig. 5A). For the stock A, only the effect of selection for OsHV-1 was significant, with higher mortality (74%) for the unselected oysters (control and VA selected groups) than the 47% mortality rate for selected oysters (OsHV-1 selected and dual selection groups) (Table 3). For stock B, a significant interaction between the selection for OsHV-1 and *V. aestuarianus* was found ($P < 0.01$). Thus, at the viral selection level, dually selected oysters had a significant lower mortality (20%) than those only selected for OsHV-1 (37%) ($P < 0.01$), while both control and VA selected groups had similar and higher mortality (75-77%) (Fig. 5A). At the bacterial selection level, Va selected and control groups both had significantly higher mortality (75% and 77%, respectively) than OsHV-1 selected and dually selected oysters (37% and 20%, respectively) ($P < 0.01$) (Fig. 5A).

From October 2014 to February 2016, the mean mortality reached 25%, ranging from 10% for the VA selected group of stock A to 52% for the control group for stock B (Fig. 5B). All factors were not significant for stock A (Table 3), although Va selected oysters had lower mortality (10%) than the control oysters (31%) (Fig. 5B). In contrast, a significant difference in mortality was found at the viral selection for stock B with higher mortality (41%) for the unselected oysters (control and Va selected groups) than the 19% for selected oysters (OsHV-

1 selected and dual selection groups) ($P < 0.01$; Table 3). Lower mortality of 24% was also observed for the selected oysters for bacterial resistance (VA selected and dual selection groups), compared to the rate of 38% for unselected oysters (control and OsHV-1 selected groups), although the difference was not significant ($P = 0.051$; Table 3).

For the final cumulative mortality in February 2016, the mean mortality among groups and stocks reached 67%, ranging from 32% for the dual selection group of stock B to 89% for the control group of stock B (Fig. 5C). For stock A, only the OsHV-1 factor was significant with lower mortality (59%) for the selected oysters (OsHV-1 selected and dual selection groups) than the rate of 80% for unselected oysters (control and Va selected groups) ($P < 0.05$; Table 3). For the bacterial selection, unselected oysters (control and OsHV-1 selected groups) and selected oysters (Va selected and dual selection groups) had similar mortality rates of 66% and 72%, respectively (Table 3). For stock B, the interaction between the viral and bacterial selection was not significant ($P = 0.10$). Oysters selected for *V. aestuarianus* (Va selected and dual selection groups) had significantly lower mortality (58%) than the rate of 71% of unselected oysters (Control and OsHV-1 selection groups) ($P < 0.01$; Table 3). Similarly, oysters selected for increased resistance to OsHV-1 (OsHV-1 selected and dual selection groups) had significantly lower mortality (40%) than the rate of 86% for unselected oysters (control and Va selected groups) ($P < 0.01$; Table 3).

3.5. Realized heritability to increase resistance to *V. aestuarianus* infection after one generation of mass selection in *C. gigas*

For trial 3, the realized heritability was not estimated for stock A due to the absence of response to selection (control vs Va selected) or a negative response to selection (OsHV-1 selected vs dual selection) (Fig. 4). In contrast, the realized heritability was moderate for the

two lines (BC and BS) of stock B (0.30 ± 0.06) when comparing the VA selected group to the control group (BC line), and 0.47 ± 0.10 when comparing the dual selection group to the OsHV-1 selected group (BS line) (Table 4). For the field exposure and stock A, a positive response to selection was only observed for the line AC for the period from October 2014 to February 2016 (Fig. 1 and 5B). The realized heritability was moderate (0.33 ± 0.01). Concerning stock B, realized heritability was estimated for both BS and BC lines for all periods. The BC line had a low realized heritability ranging from 0.05 to 0.18, while a much higher value was found for the BS line, ranging from 0.47 to 0.80 (Table 4).

3.6. OsHV-1 and *V. aestuarianus* detection

For trial 1, all moribund oysters were positive for the detection of *V. aestuarianus*, with a high amount of bacterial DNA (>10^5 copies for 25 ng of total DNA; Table 5). Also, OsHV-1 was detected for 16% of the samples analyzed and all at a low level with <10^2 copies for 25 ng of total DNA (Table 5). Similar results were found for trial 3. For trial 2, all oysters examined were positive for high concentrations of OsHV-1 DNA (>10^5 copies of OsHV-1 DNA per 25 ng of total DNA), but none were positive for *V. aestuarianus*.

For the field exposure, all oysters examined were positive for high concentrations of OsHV-1 DNA (>10^5 copies of OsHV-1 DNA per 25 ng of total DNA) during the main peak of mortality in June. In addition, 31% of the moribund oysters were also positive for *V. aestuarianus*, although the amount of the bacterial DNA was lower (10^3 copies per 25 ng of total DNA) (Table 5). In April 2015, the moribund oyster was positive only for *V. aestuarianus* at a high level (>10^5 copies per 25 ng of total DNA) (Table 5).

4. Discussion

Both OsHV-1 and *V. aestuarianus* have reduced the French oyster production since 2008. For both pathogens, a genetic basis for resistance has been demonstrated, in particular for the resistance of OsHV-1 (Azéma et al., 2017b; Dégremont et al., 2015c). The lack of genetic correlations between resistance to OsHV-1 infection and resistance to *V. aestuarianus* infection indicates that selection to improve resistance to OsHV-1 infection should neither increase nor decrease the resistance to *V. aestuarianus* infection. The observations also indicate the possibility to select for dual resistance in *C. gigas* (Azéma et al., 2017b). Selection for dual resistance in *C. gigas* could then be conducted by commercial hatcheries, which have mainly focused on OsHV-1. Our study provides the first findings on a dual selection using two stocks of oysters that were either unselected or selected for resistance to one or both pathogens, and which were both tested using experimental infections and in the field to validate the potential value of interest for farmers previously demonstrated by Dégremont et al. (2010a).

The main finding of this study is the positive response to selection after one generation of mass selection to increase the resistance to *V. aestuarianus* for stock B when oysters were tested at the adult size under experimental infection (trial 3 – Fig. 3) as well as under field exposure in conditions where the bacteria have been routinely detected since 2012 (Fig. 4, Table 4) (Azéma et al., 2017a; Dégremont et al., 2019). The gain of survival after one generation of selection for stock B was 12% and 17% for the group selected for dual resistance and the group only selected for *V. aestuarianus*, respectively, after 16 days of exposure to *V. aestuarianus* in trial 3 (Fig. 4). The gain of survival after 2 years of growth under field exposure was 5% for oysters selected only for *V. aestuarianus* and 21% for oysters selected for dual resistance (Fig. 5C). Such genetic gains are common in fish and shellfish species, as recently reviewed by Gjedrem and Rye (2018), in particular for disease

resistance in oyster species (Dégremont et al., 2015a). A low or absent genetic variation for bacterial resistance in stock A could explain the absence of a response to selection, especially when the intensity of selection was low (Table 1). To support this hypothesis, the surviving G3 oysters for stocks A and B, which produced the G4 groups selected for the resistance to the bacteria (Va selected and dual resistance) (Fig. 1), were challenged twice with *V. aestuarianus* after spawning. While the mortality rates of the AC and BC lines exceeded 99%, the final cumulative survival was much lower for the oysters of the AS line (4%) than for the oysters of the BS line (16%) (Azéma et al., 2015), suggesting a lower resistance to the bacteria for the stock A than stock B for oysters previously selected for their resistance to OsHV-1. The absence of resistance to the bacteria in stock A might be explained by the genetic composition of the initial population sampled in the wild. Alternatively, this resistance, if it existed, was intentionally lost through genetic drift over the three successive generations of reproduction, regardless of the selection applied for OsHV-1 resistance, as both AC and AS lines showed a similar mortality pattern when exposed to the bacteria (Azéma et al., 2015).

Our study is the first to report the realized heritability for resistance to *V. aestuarianus*. It was not possible to estimate this parameter in trial 1 in contrast with trial 3. This could be explained by a higher susceptibility to the bacteria for older and/or larger oysters as previously demonstrated (Azéma et al., 2016; Azéma et al., 2017b). Although in trial 1 oysters tested at 12 g were more susceptible to *V. aestuarianus* than spat size oysters (3 g) (Fig. 1), only the adult stage was appropriate to estimate the realized heritability. In trial 3, in which adult oysters were challenged, the moderate (0.30) to high (0.47) realized heritability for the BC and BS oysters, respectively (Table 4), agreed with the narrow sense heritability for survival of *C. gigas* when exposed to *V. aestuarianus* at the adult stage under laboratory conditions ($h^2 = 0.33$) (Azéma et al., 2017b). In the field condition, oysters were exposed to

pathogens naturally present in the environment, in particular OsHV-1 and *V. aestuarianus*. The realized heritability to increase resistance for *V. aestuarianus* was lower in the field than in trial 3 for the BC stock (Table 4), indicating a lower response to selection as the intensity of selection was constant. This could be explained by the high mortality due to OsHV-1 in the first growing year (75-77%) (Fig. 5A) or a longer exposure to *V. aestuarianus* in the field than in the laboratory.

In contrast, heritability for resistance to *V. aestuarianus* was higher in the field than in trial 3 for the BS oysters (Table 4). It is conceivable that primary exposure to OsHV-1 enhances the response to selection for *V. aestuarianus* for dual selection. Considering the presence of virus and bacteria, in both infections, pathogenesis involved a hemolymphatic initial colonization step, which is considered a key step determining oyster outcome (Martenot et al., 2017; Parizadeh et al., 2018). Thus, OsHV-1 exposure might have initiated cellular and molecular immune responses, conferring protection against other pathogens (Green and Montagnani, 2013). Using a holistic approach, a recent study demonstrated that oysters able to survive to OsHV-1 share a particular early immune response reprogrammed to kill opportunistic bacteria that have also been implicated in mortality outcomes (de Lorgeril et al., 2018). However, this antimicrobial immunological control still has to be linked to *V. aestuarianus*.

In our study, the field exposure from spat to market-size oysters mimicked the farming practices used by the French oyster farmers. Thus, spat mainly suffered from OsHV-1 during their first year, since oysters selected for their higher resistance to the virus for both stocks showed significant reduction of the mortality over the control oysters, regardless their level of selection to the bacteria, with values of -27% and -49% in October 2014 for stock A and B, respectively (Table 3, Fig. 5A). These results agree with the genetic gain obtained for each stock of the fourth generation of mass selection to increase resistance to OsHV-1 infection (Dégremont et al., 2015b). Also, and only for BS oysters (Fig. 1), those selected for dual

resistance had lower mortality (-17%) than those only selected for OsHV-1 resistance (Fig. 5A). *V. aestuarianus* was detected along with OsHV-1 in moribund oysters sampled in June 2014 (Table 5), and also for other oyster groups with similar life-history and sampled from June to September 2014 (Azéma et al., 2017a). This suggests that both pathogens were active in our experimental farms in 2014, and that selection for dual resistance to OsHV-1 and *V. aestuarianus* was successful at the spat/juvenile stage for stock B as the oysters weighed 2.4 g at deployment in December 2013 and 41.8 g in October 2014. From October 2014 to endpoint, oysters selected for *V. aestuarianus* (Va and dual) had lower mortality than unselected oysters (-9 and -14% for stock A and B, respectively) (Table 3), and oysters selected for OsHV-1 (OsHV-1 and dual) had lower mortality than their respective controls (-22%) only for stock B (Table 3, Fig. 5B). Both pathogens could be involved in the mortality observed during this period with a predominant impact of the bacteria. Unfortunately, only one moribund oyster was analyzed and was positive only for *V. aestuarianus*. At the endpoint, breeding for resistance to both diseases was effective for stock B with mortality decreasing from 89% for the unselected control, to 84% for oysters selected for their resistance to the bacteria, to 53% for the oysters selected only to OsHV-1, and to 32% for those selected for dual resistance (Fig. 5C). Similar findings were reported for dual resistance in oysters as in *C. virginica* against *Haplosporidium nelsoni* and *Perkinsus marinus* with a decrease in mortality reaching 40-45% (Dégremont et al., 2012; Ragone Calvo et al., 2003), and in *Saccostrea glomerata* against *Marteilia sydneyi* and *Bonamia roughleyi* with a decrease in mortality reaching 35% for the oysters selected for dual resistance in comparison to unselected oysters (Dove et al., 2013). Finally, selection should first be carried out based on the resistance to OsHV-1 infection before selecting for resistance to *V. aestuarianus* infection. The virus is the main *C. gigas* pathogen in France with mortality reaching 70-90% each year for unselected stocks, mainly affecting younger animals (spat-juveniles) (Azéma et al., 2017b). In contrast,

the *V. aestuarianus* mostly affects older animals (juveniles-adults) (Azéma et al., 2017b), so selection for resistance to the bacteria only would have a very limited impact on oyster mortality, since most oysters will die from the virus.

5. Conclusion

The selective breeding program to enhance dual resistance to OsHV-1 and *V. aestuarianus* was effective for stock B. The response to selection to enhance the resistance to the bacteria was only observed in *C. gigas* adults after 16 days of exposure in controlled experimental infection using oysters selected only for the bacterial resistance as well as in oysters selected for dual resistance. In the field evaluation, such a response was observed only for oysters selected for dual selection after the first growing season, and to a lesser extent during the second growing season for oysters only selected for bacterial resistance. The breeding program should deal with the resistance to OsHV-1 first, and then focus on dual resistance by adding the resistance to *V. aestuarianus*. Such a strategy could greatly enhance oyster aquaculture production. Further investigations are required, especially in triploid oysters which represent 80-90% of the French hatchery production. To achieve this goal, selective breeding of oysters for dual resistance should be conducted in both diploid and tetraploid oysters.

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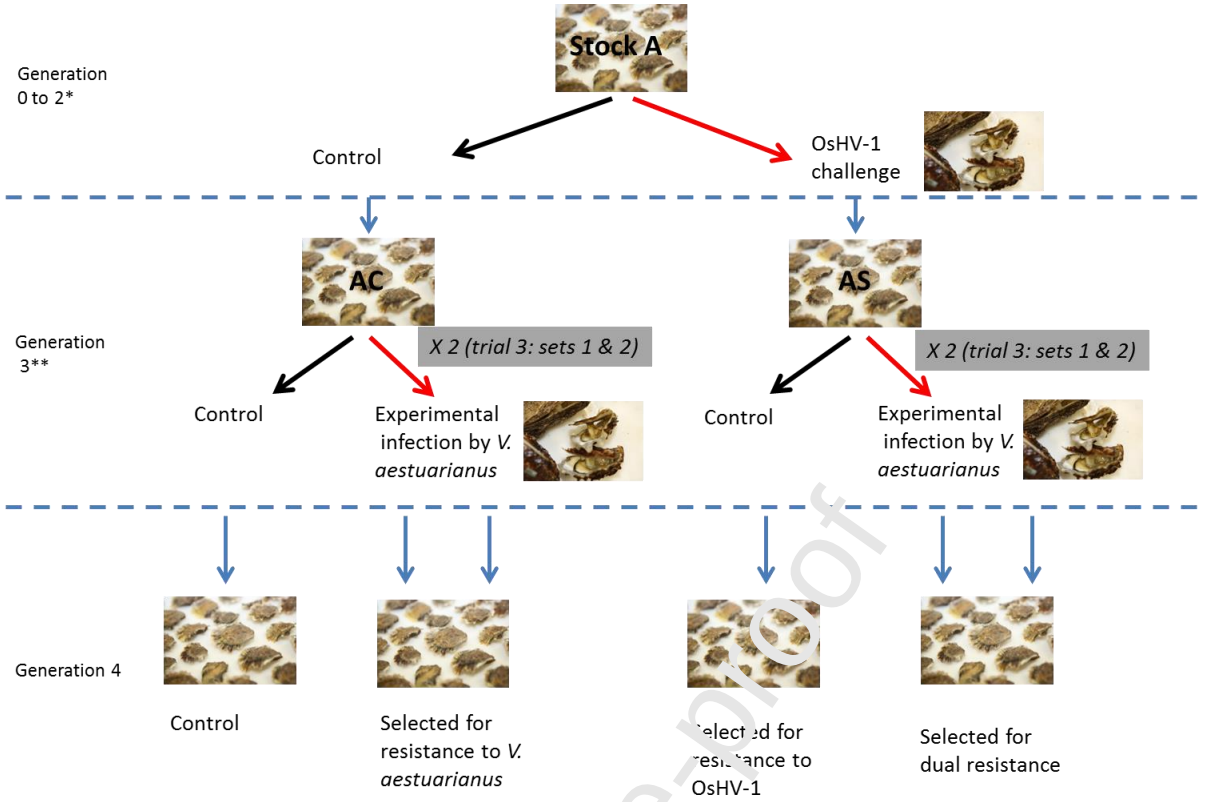


Figure 1: Summary of the production of the control group, the group selected for resistance to *V. aestuarianus*, the group selected for resistance to OsHV-1, and the group selected for dual resistance of stock A tested in this study (generation 4) (the same approach was used for stock B). * Details of the experiments for the OsHV-1 challenge until generation 2 are given in Dégremont et al. (2015). ** Details of the experiments for the experimental infection by *V. aestuarianus* of the generation 3 are given for the sets 1 and 2 of the trial 3 in Azéma et al. (2015). A black arrow indicates no exposure to pathogens, while a red arrow indicates an exposure to a pathogen. A blue arrow indicates a spawning event using naïve oysters or survivor oysters, and so the dotted blue lines separate the generations.

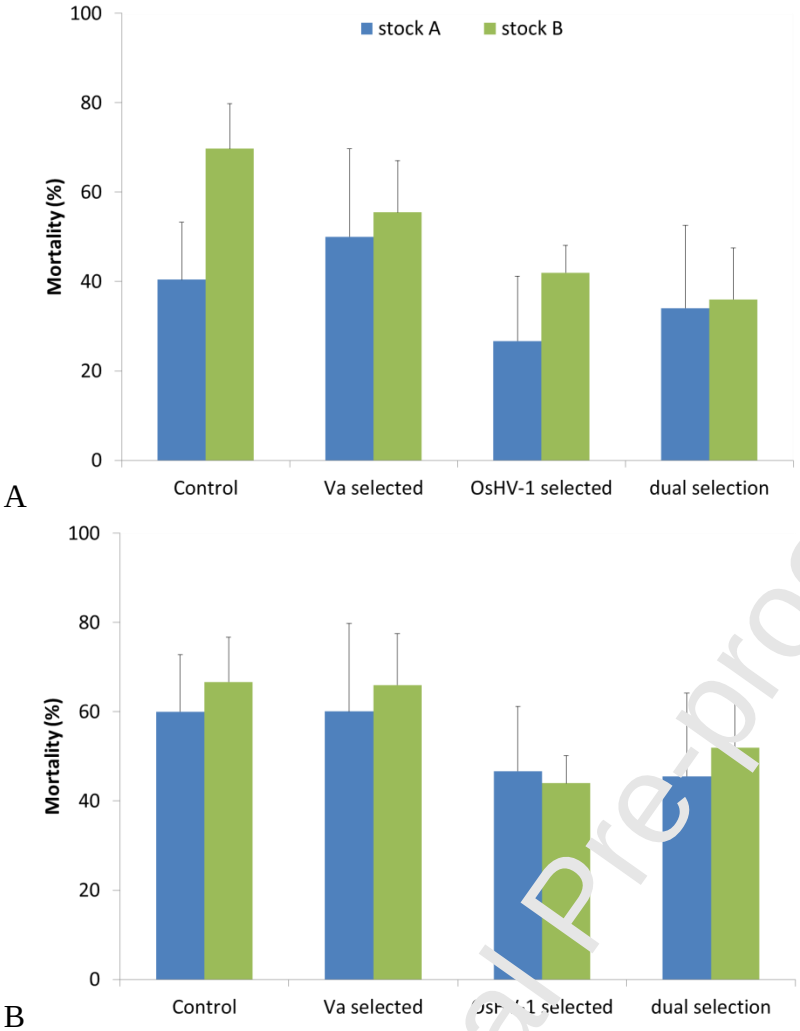
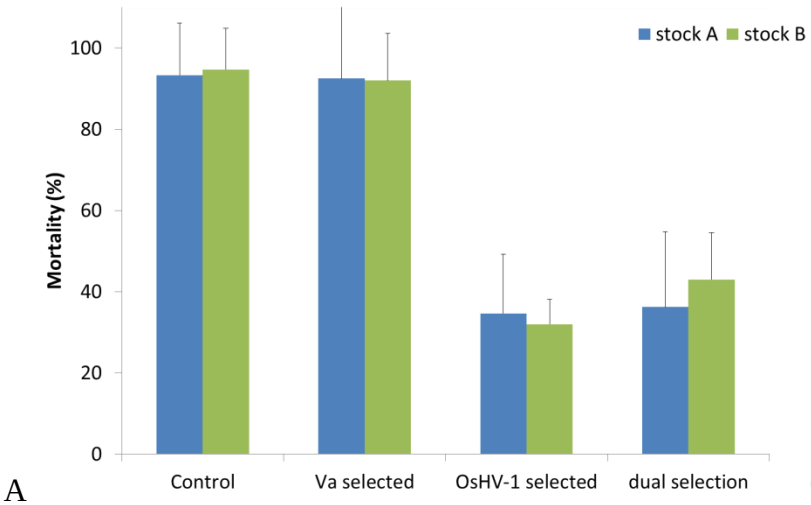
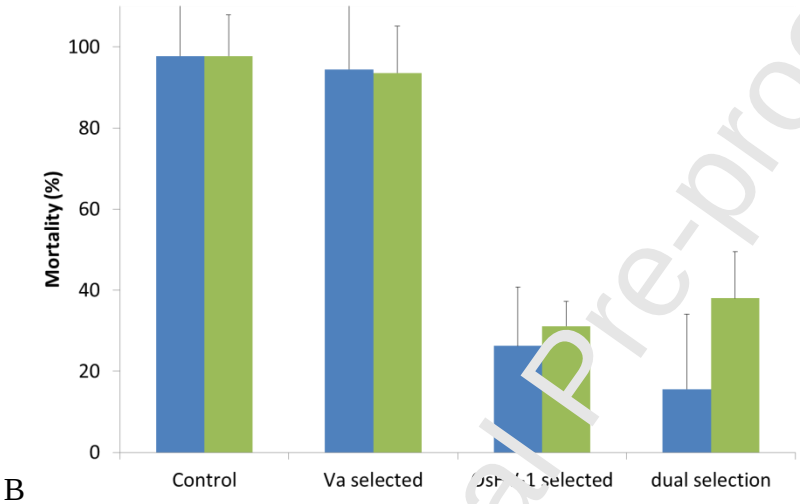


Figure 2: Mortality (% + standard deviation among replicates) 12 days post-infection by *V. aestuarianus* for the four groups of stock A (in blue) and B (in green) in trial 1. Top panel A: spat size (mean individual weight 3 g), and bottom panel B: juvenile size (mean individual weight 12 g).



A



B

Figure 3: Mortality (% + standard deviation among replicates) 12 days post-infection by *OsHV-1* for the four groups of stock A (in blue) and B (in green) in trial 2. Top panel A: spat size (mean individual weight 3 g), and bottom panel B: juvenile size (mean individual weight 12 g).

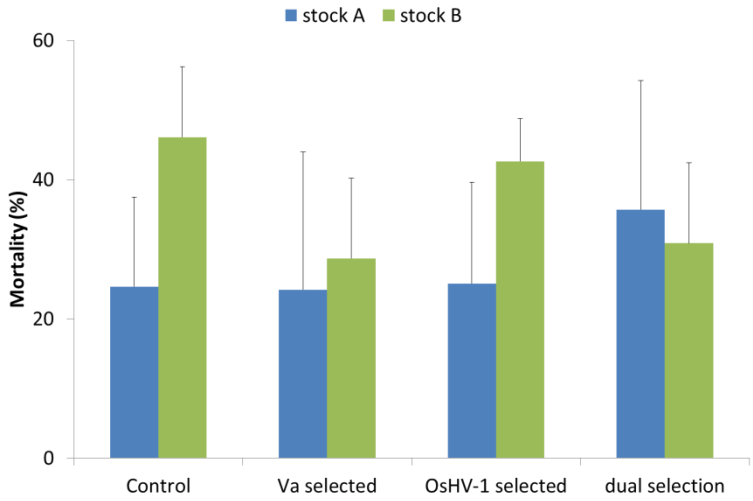


Figure 4: Mortality (% + standard deviation among replicates) 16 days post-infection by *V. aestuarianus* for the four groups of stock A (in blue) and B (in green) in *C. gigas* adults (mean individual weight 47g) in trial 3.

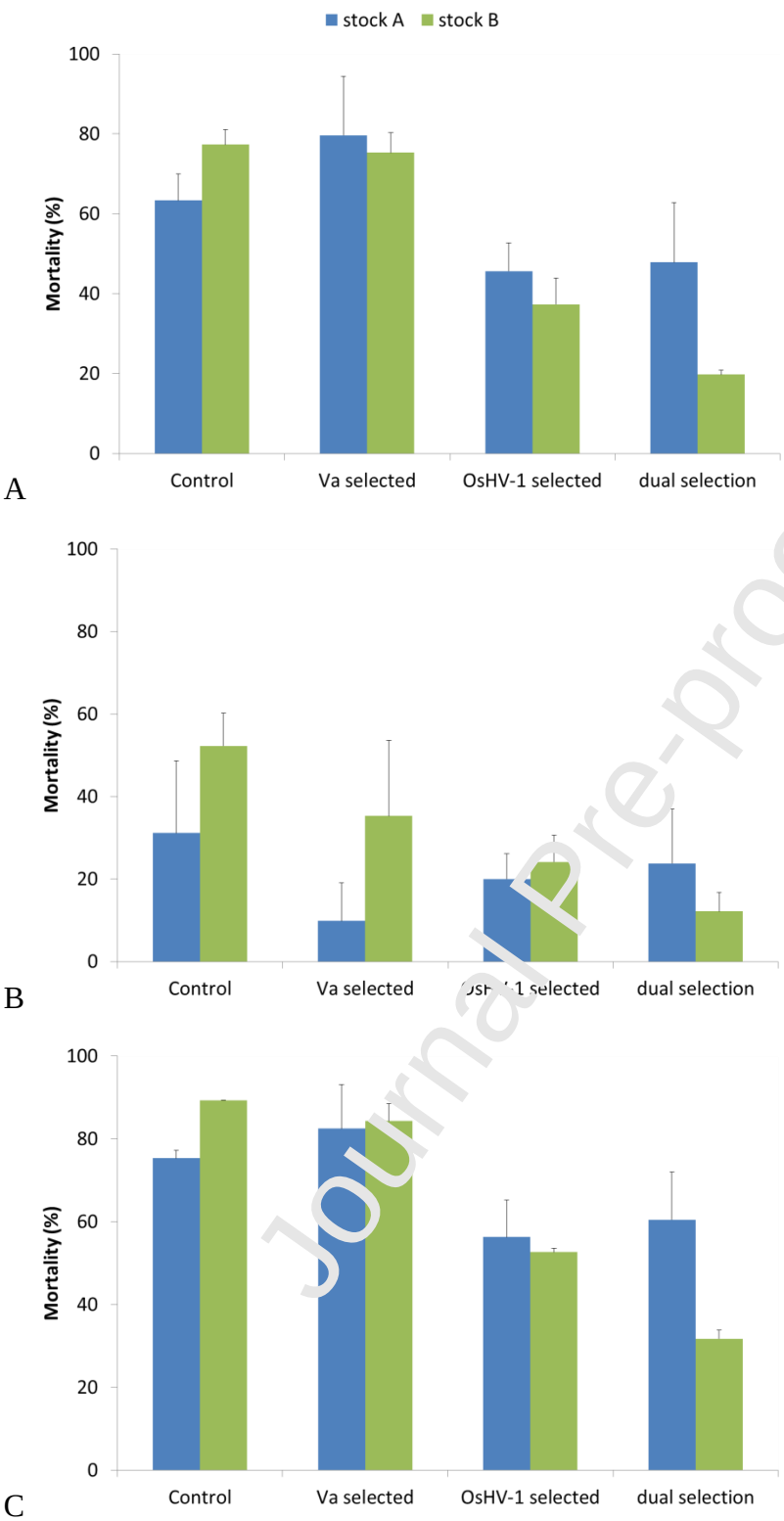


Figure 5: Mortality (% + standard deviation among replicates) for the four groups of stock A (in blue) and B (in green) in the field at Agnas. Top panel A: from December 2013 to October 2014, middle panel B: from October 2014 to February 2016, and lower panel C: cumulative mortality from December 2013 to February 2016.

Table 1 Summary of the oyster groups used as parents and their progenies

Experimental infection by *V. aestuarianus*	Trial[1]	Set[1]	Stock	Line[2]	Survival (%)[3,4]	Intensity of selection[4,5]	Number of parents used[4]	Select OsHV
cohabitation	3	1/2	A	AC	27/30	1.22/1.16	20/15	No
cohabitation	3	1/2	A	AS	61/84	0.63/0.29	46/42	Yes
cohabitation	3	1/2	B	BC	22/22	1.36/1.36	13/11	No
cohabitation	3	1/2	B	BS	56/68	0.70/0.53	42/34	Yes
none			A	AC			34	No
none			A	AS			34	Yes
none			B	BC			34	No
none			B	BS			34	Yes

[1] The trials and sets are those used in Azéma et al. (2015),

[2] The first letter corresponds to the stock (A or B), and the second letter corresponds to the level of selection to increase the resistance to OsHV-1 (C for control and S for selected),

[3] The survival is given after one experimental infection by *V. aestuarianus* and until the spawn of the survivors,

[4] The first and the second numbers are the first and second sets, respectively.

[5] The intensity of selection is the mean deviation of individuals with value exceeding the threshold. See Table A of the appendix tables in Falconer and Mackay (1996),

[6] No indicates the absence of selection for disease resistance, while Yes indicates either third generation of mass selection to increase resistance to OsHV-1 infection or first generation of mass selection to increase resistance to *V. aestuarianus* infection.

Table 2 Logit analysis of mortality within stock at endpoint for the experimental infection of trials 1 to 3

Trial	Pathogen tested	Stock	Source[1]	χ^2	P	Description[1]	U
1	*V. aestuarianus*	A	Aestu	1.06	0.30	Unselected vs Selected	43
			OsHV_1	8.10	<0.01	Unselected vs Selected	53
			Size	8.08	<0.01	Juvenile vs spat	
			OsHV_1*Aestu	0.01	0.91		
		B	Aestu	1.93	0.17	Unselected vs Selected	56
			OsHV_1	33.38	<0.01	Unselected vs Selected	63
			Size	4.16	0.04	Juvenile vs spat	
			OsHV_1*Aestu	1.54	0.21		
2	OsHV-1	A	Aestu	0.45	0.50	Unselected vs Selected	63
			OsHV_1	204.38	<0.01	Unselected vs Selected	94
			Size	3.97	0.04	Juvenile vs spat	
			OsHV_1*Aestu	0.06	0.81		
		B	Aestu	0.18	0.68	Unselected vs Selected	64
			OsHV_1	122.31	<0.01	Unselected vs Selected	94
			Size	0.04	0.84	Juvenile vs spat	
			OsHV_1*Aestu	1.48	0.22		
3	*V. aestuarianus*	A	Aestu	0.79	0.37	Unselected vs Selected	25
			OsHV_1	0.97	0.32	Unselected vs Selected	24
			OsHV_1*Aestu	0.77	0.38		
		B	Aestu	4.90	0.03	Unselected vs Selected	44
			OsHV_1	0.01	0.94	Unselected vs Selected	35
			OsHV_1*Aestu	0.18	0.67		

[1] Aestu: test for difference between unselected oysters (Control and OsHV-1 selected groups) and selected oysters to increase *V. aestuarianus* resistance (*V. aestuarianus* selected and dual selection groups)

[1] OsHV-1: test for difference between unselected oysters (Control and *V. aestuarianus* selected groups) and selected oysters to increase OsHV-1 resistance (OsHV-1 selected and dual selection groups)

Table 3 Logit analysis of mortality within stock for the field study

Date	Stock	Source[1]	χ^2	P	Description[1]	U
Dec.13 to Oct. 14	A	Aestu	1.41	0.23	Unselected vs Selected	55
		OsHV_1	8.39	<0.01	Unselected vs Selected	74
		OsHV_1*Aestu	0.92	0.34		
	B	Aestu	12.28	<0.01	Unselected vs Selected	57
		OsHV_1	253.98	<0.01	Unselected vs Selected	76
		OsHV_1*Aestu	7.20	<0.01		
Oct. 14 to Feb.16	A	Aestu	0.82	0.36	Unselected vs Selected	26
		OsHV_1	0.00	1.00	Unselected vs Selected	17
		OsHV_1*Aestu	2.33	0.13		
	B	Aestu	3.81	0.051	Unselected vs Selected	38
		OsHV_1	13.68	<0.01	Unselected vs Selected	41
		OsHV_1*Aestu	0.01	0.93		
Dec.13 to Feb.16	A	Aestu	0.91	0.34	Unselected vs Selected	66
		OsHV_1	10.32	<0.01	Unselected vs Selected	80
		OsHV_1*Aestu	0.17	0.68		
	B	Aestu	27.03	<0.01	Unselected vs Selected	71
		OsHV_1	363.34	<0.01	Unselected vs Selected	86
		OsHV_1*Aestu	2.64	0.10		

[1] Aestu: test for difference between unselected oysters (Control and OsHV-1 selected groups) and selected oysters to increase *V. aestuarianus* resistance (Va selected and dual selection groups)

[1] OsHV-1: test for difference between unselected oysters (Control and *V. aestuarianus* selected groups) and selected oysters to increase OsHV-1 resistance (OsHV-1 selected and dual selection groups)

Table 4 Realized heritability to increase the resistance to *V. aestuarianus* infection after one generation of mass selection in *C. gigas* for the trial 3 and the field study

Stock	A	
Line	AC	AS
Group unselected oysters for *V. aestuarianus*	Control	OsHV-1 selected
Group selected oysters for *V. aestuarianus*	*V. aestuarianus* selected	Dual selection
Trial 3	NC	NC
Field December 2013 to October 2014	NC	NC
Field October 2014 to February 2016	0.33 ± 0.01	NC
Field December 2013 to endpoint	NC	NC

NC: not calculated due to the absence of response to selection (i.e. unselected group had a lower mortality than the selected group).

Table 5: Detection and quantification (DNA copies per 25 ng of total DNA) of OsHV-1 and *Vibrio aestuarianus* DNA in moribund oysters of sampled during the experimental infections (trials 1 to 3) and the field evaluation.

	Date	OsHV-1			*V. a*
		Positive/analyzed	Prevalence (%)	Quantification	Positive/analyzed Pre
Trial 1	May 2014	3/18	16	$<10^2$	18/18
Trial 2	June 2014	18/18	100	$>10^5$	0/18
Trial 3	January 2015	4/30	13	$<10^2$	29/30
Field	June 2014	13/13	100	$>10^5$	4/13
Field	April 2015	0/1	0		1/1

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Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

- This is the first investigation of mass selection to enhance resistance to *Vibrio aestuarianus* in *Crassostrea gigas.*
- Only one stock showed a positive response to selection.
- Stock B showed a gain of survival of +14% for experimental infection at the adult stage.
- Dual selection for OsHV-1 and the bacteria increased the field survival of *C. gigas* from 11% to 68% for stock B.