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Susceptibility variation to the main pathogens of *Crassostrea gigas* at the larval, spat and juvenile stages using unselected and selected oysters to OsHV-1 and/or *V. aestuarianus*

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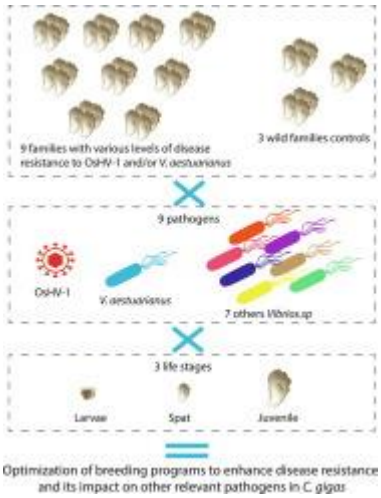
Abstract :

French commercial hatcheries are massively producing Crassostrea gigas selected for their higher resistance to OsHV-1, and soon should also implement selection for increasing resistance to Vibrio aestuarianus. The first objective of this study was to optimize the breeding programs for dual resistance to OsHV-1 and V. aestuarianus to determine the earliest life stage for which oysters are able to develop disease resistance. Wild stocks and selected families were tested using experimental infections by both pathogens at the larval, spat and juvenile stages. Oyster families could be evaluated for OsHV-1 as soon as the larval stage by a bath method, but this only highlighted the most resistant families; those that showed the highest resistance to V. aestuarianus could be determined using the cohabitation method at the juvenile stage.

The second objective of this study was to determine if selection to increase/decrease the resistance to OsHV-1 and V. aestuarianus could have an impact on other major pathogens currently detected in hatchery at the larval stage, and in nursery and field at the spat/juveniles stages (V. coralliilyticus, V. crassostreae, V. tasmaniensis, V.neptunius, V. europaeus, V. harveyi, V. chagasi). No relationship was found between mortality caused by V. aestuarianus/OsHV-1 and the mortality caused by the other virulent bacterial strains tested regardless the stages, except between OsHV-1 and V. tasmaniensis at the juvenile stage.

Finally, miscellaneous findings were evidenced such as (1) bath for bacterial challenges was not adapted for spat, (2) the main pathogens at the larval stage were OsHV-1 and V. coralliilyticus using bath, while it was V. coralliilyticus, V. europaeus, and V. neptunius at the juvenile stage by injection, and (4) variation in mortality was observed among families/wild controls for all pathogens at larval and juvenile stages, except for V. harveyi for larvae.

Graphical abstract



Highlights

► From the 12 families/wild stocks tested, only one was resistant to OsHV-1 from larvae to juvenile, ► No mortality by *V. aestuarianus* using bath for oysters lesser than 5 mm, ► Breeding programs on disease resistance should now focus on *V. coralliilyticus* which induced moderate mortality in larvae and high mortality in juveniles, ► No impact of the selection for OsHV-1 and/or *V. aestuarianus* resistance to other virulent strains of *Vibrios* of *C. gigas,* except for *V. tasmaniensis.*

Keywords : *Crassostrea gigas*, OsHV-1, *V. aestuarianus, Vibrio spp*, selection

1. Introduction

Selective breeding to enhance disease resistance has been successful in many oyster species (Dégremont et al., 2015a). Usually most of the studies investigate one pathogen, such as *Roseovarius crassostrea* in *Crassostrea virginica* (Barber et al., 1998) or *Bonamia ostreae* in *Ostrea edulis* (Culloty et al., 2001; Naciri-Graven et al., 1998); or two pathogens, as described for *Perkinsus marinus* and *Haplosporidium nelsoni* in *C. virginica* (Frank-Lawale et al., 2014; Ragone Calvo et al., 2003), *Bonamia roughleyi* and *Marteilia sydneyi* in *Saccostrea glomerata* (Dove et al., 2013), and the ostreid herpesvirus type 1 (OsHV-1) and *Vibrio aestuarianus* in *Crassostrea gigas* (Azéma et al., 2015).

Since 2008, French oyster production, mostly based on *C. gigas*, has been threatened by the Ostreid herpesvirus 1 (OsHV-1) with high mortality rates of over 70% for spat and juveniles. In addition, market-size adults have displayed mortality due to the bacterium *Vibrio aestuarianus* since 2012 (Azéma et al., 2015). As a response, selective breeding programs have been developed to enhance as well as to decrease OsHV-1 resistance at the spat stage in *C. gigas* (Dégremont, 2011; Dégremont et al., 2015b), and such a strategy is currently under investigation for *V. aestuarianus*, based on the results obtained by Azéma et al. (2017b) who showed a low to moderate genetic basis for resistance to this pathogen at juvenile and adult stages. More importantly, they found the absence of genetic correlation between the resistance to OsHV-1 infection and the resistance to *V. aestuarianus* infection, suggesting that it could be possible to develop oyster lines in *C. gigas* with either resistance to one of the two diseases, dual susceptibility, or dual resistance to OsHV-1 and *V. aestuarianus*. Nevertheless, such selection could alter the susceptibility of *C. gigas* to other pathogens, such as those frequently detected during mortality outbreaks in hatcheries and nurseries, if a negative or positive genetic correlation

exists, as observed in rainbow trout between *Piscirickettsia. salmonis* resistance and *Caligus rogercresseyi* susceptibility (Bassini et al., 2019), and between resistance to *Flavobacterium psychrophilum* and resistance to *Flavobacterium columnare* (Silva et al., 2019), respectively.

Beside OsHV-1 and *V. aestuarianus*, other pathogens can induce high mortality in *C. gigas*. For instance, *Vibrio coralliilyticus* and *Vibrio neptunius* causes high larval mortality (Prado et al., 2005; Richards et al., 2015). Similarly, larvae, juvenile and adults showed high mortality due to *Vibrio tubiashii* and the close relative *Vibrio europaeus* (Dubert et al., 2016; Elston et al., 2008; Mersni-Achour et al., 2015; Travers et al., 2014). Spat and juveniles are also susceptible to *Vibrio harveyi* (Saulnier et al., 2010), *Vibrio crassostreae* (Bruto et al., 2017), and *Vibrio splendidus*-related LGP32 (Gay et al., 2004), the latter being assigned to *V. tasmaniensis* (Sawabe et al., 2013).

Our study investigates the susceptibility of nine families selected to decrease or increase their resistance to OsHV-1 and/or *V. aestuarianus* and three wild stocks at the larval, spat and juvenile stages. The 12 groups of oysters were challenged with OsHV-1 and *V.aestuarianus*, as well as *V. coralliilyticus, V. crassostreae, V. tasmaniensis, V. harveyi, V.neptunius,* and *V. europaeus.* In addition, juvenile oysters were also tested against *V. chagasii*. The two main objectives were (1) to determine which stage was the best to identify to the susceptibility or the resistance for OsHV-1 and *V. aestuarianus*, and (2) to evaluate if any relationship exists in performance under challenge with one of these two pathogens and with other pathogenic strains of *Vibrio*.

2. Materials and methods

2.1. Oyster broodstocks

Three wild stocks collected from the Marennes-Oléron Bay in January 2016 and nine families with various levels of susceptibility to OsHV-1 and *V. aestuarianus,* the latter named from F1 to F9, were used (Table 1). The level of susceptibility of the families was defined at the juvenile stage for oysters weighing around 10-15g, and using a cohabitation method with donors injected with one of the two pathogens. Eight (F1 to F8) of them were produced from wild oysters in March 2013 and their disease susceptibility was evaluated as described in Azéma et al. (2017b). The last family (F9), produced in February 2014, used a female of the family F8 crossed with a male from the third generation of a mass selection scheme to increase OsHV-1 resistance as described in Dégremont et al. (2015b). No family had a common ancestor, meaning that the inbreeding rate (F) is assumed to be null, as it was for the three stocks of wild oysters sampled in the Marennes-Oléron Bay (Table 1).

2.2. Oyster production

In January 2016, the nine families and the three wild stocks were placed in separate tanks in the conditioning room at the Ifremer hatchery in La Tremblade until the spawn. Indeed, wild stocks may have contained asymptomatic adults infected with OsHV-1, *V. aestuarianus* or other pathogens. Thus, separate holding avoided potential horizontal transmission of pathogens from the wild stocks to the families, in particular the susceptible ones, as they were always protected in our facilities using UV-treated seawater (40 mj/cm2), and so they never experienced mortality

outbreaks. The seawater temperature was increased and maintained to 20°C, and a cultured phytoplankton diet (*Isochrysis galbana, Tetraselmis suecica* and *Skeletonema costatum*) was provided to favor gametogenesis.

The spawn occurred in March 2016. For each of the families and wild stocks, oysters were placed into a 5-L glass beaker filled alternatively with unheated or heated seawater to 28°C to induce the spawn. The number of oysters used per stock is reported in Table 1. As soon as the first gametes were released, seawater was maintained at 25°C. Every 15 minutes, 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 in order for them to continue to spawn. This occurred until the end of the spawning event to maximize the effective population size for each stock.

The methodology used for the larval 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 UV-treated seawater until their evaluations either under experimental infections by *V. aestuarianus* or OsHV-1 or under field conditions where both pathogens have been regularly detected in moribund oysters (Azéma et al., 2017a).

2.3. Preparation of viral suspension and contaminated seawater

Viral suspensions were prepared according to Schikorski et al. (2011). In brief, gills and mantles from OsHV-1 µVar-infected oyster spat were dissected and pooled together in a 50-mL sterile tube. All subsequent dilutions were made with 0.22-µm-filtered artificial seawater (ASW). The total mass of the tissues was weighed, and 10 volumes of 0.22-µm ASW were added to the tube (9 mL of seawater per g of tissue). The tissues

were then crushed on ice with an Ultra-Turrax® mixer (3×5 s). Following centrifugation (1000 g, 5 min, 4°C), the supernatant was placed in a new tube and diluted by adding four volumes of ASW. Finally, the clarified tissue homogenate was filtered consecutively through syringe filters at 5, 2, 0.45, and 0.22-µm pore sizes under sterile conditions. Filtered tissue homogenates were stored at 4°C until use and the concentration of the viral suspension obtained was 3.3 x 10^5 μl^{-1} copies of viral DNA.

For the experimental infections at the larval stage, 100 µl of viral suspension was added to each well corresponding to a final concentration of 10^4 OsHV-1 DNA copies μl^{-1}. For the experimental infections at the spat and juvenile stages, contaminated seawater was prepared through injection of 12 oysters with 100 µl of the viral suspension. The injected oysters were maintained in a 3-L tank filled with UV-treated, filtered and aerated seawater for 24 h. Contaminated seawater surrounding oysters was added to each well for spat (4.7 x 10^3 DNA copies μl^{-1}) or in tanks for juveniles (5.3 x 10^3 DNA copies μl^{-1}).

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2.4. Preparation of contaminated seawater for several pathogenic strains of *Vibrio*

Bacteria were initially grown on Zobell agar (peptone 4 g/L, yeast extract 1 g/L, Tris buffer 0.5 g/L in artificial seawater) from stock cultures, and stored at -80°C in Zobell containing 15% glycerol (v/v). After a few days, one colony was grown at 22°C for 20 h in Zobell broth, with constant shaking at 40 rpm (Rotator SB3; Stuart). The cells were centrifuged at 3,200 g for 10 min, the supernatant discarded and the resulting pellet resuspended in SASW to obtain an OD600 nm = 1. Purity and concentration of all suspensions were checked by plating, corresponding to 1 x 10^9 bacteria/ml, except for *V. aestuarianus*, for which it corresponded to 5 x 10^8 bacteria/ml.

For a bath protocol at larvae stage, bacteria were directly diluted in seawater containing larvae to reach a final concentration of 10^5 bacteria ml^{-1}. For bath protocol at spat stage, bacteria were first injected in muscle of $MgCl_2$ anesthetized oysters (15 oysters/strain with 100 µl of bacterial suspensions (DO600nm=1)). Those oysters were conserved in 3 L of UV-treated, filtered and aerated seawater for 24 h. Contaminated seawater surrounding oysters was diluted 1/20 in fresh UV-treated seawater, corresponding to 2 to 5 x 10^5 freshly shed bacteria ml^{-1}. Bacterial viability and concentration were checked by plating and qPCR (Pollock et al., 2010; Saulnier et al., 2017; Travers et al., 2014).

Bacterial isolates used here (Table 2) are already recognized as oyster pathogens and were clearly affiliated to the different *Vibrio* species. However, we also used a new *V. neptunius* isolate (09-123_1T2), identified here through 16S rDNA sequencing (GenBank number MT298104) and the targeted PCR designed by Lago et al. (2009).

2.5. Experimental infection

Five experimental infections were carried out for all families and wild stocks. Two occurred at the larval stage, trials Larval 1 (size 100 µm) and Larval 2 (size 150 µm); two at the spat stage, trials Spat 1 (size 5 mm) and Spat 2 (size 5 mm); and one at the juvenile size (size 50-60 mm, 20g) (Table 3).

2.5.1. Experimental infection with OsHV-1

For each experimental infection, the bath method with contaminated seawater was used. For the larval stage, six-well plates were used using three wells per family and wild stock. Each well contained 5 ml of contaminated seawater and approximately 100 larvae per well (+/- 30%) (Table 3).

Similarly, three wells per family and wild stock were used as controls using seawater that was filtered and UV-treated (40 mj/cm2). As a plate contained six wells, no plates contained both contaminated and control conditions, to limit unintentional contamination of the control. Plates were stored in an incubator at 20°C, without movement, air bubbling and food. Mortality was recorded at days 2, 5 and 7 post-infection by placing the six-well plate under a binocular magnifier and by counting dead and live individuals for the first 30 larvae observed.

For the spat stage, similar protocols were used with the exception that six wells were used per family and wild stock, containing 5 spat each (2-10 mm) in 10 ml of contaminated seawater or filtered and UV-treated seawater (Table 3). Mortality was counted at days 4 and 7 post infection for the first challenge, and at days 5 and 7 for the second. At days 4 and 5, dead oysters were removed from the wells.

Finally, three tanks were used per family and wild stock each containing 10 juveniles in 3 liters of contaminated seawater with aeration (Table 3). Mortality was recorded daily until day 7 post-infection, and dead oysters were removed each day. For the control, only one tank per family was used.

2.5.2. Experimental infection with several pathogenic strains of *Vibrio* in *C. gigas*

Bacteria were grown on Zobell agar (peptone 4 g/L, yeast extract 1 g/L, Tris buffer 0.5 g/L in artificial seawater) from stock cultures, stored at -80°C in Zobell containing 15% glycerol (v/v). The same protocols as described above with OsHV-1 were also used for each of the following bacteria (Table 2): *V. aestuarianus 02/041, V. coralliilyticus 06/210, V. crassostreae J2-9, V. harveyi 08/076_3T1, V. tasmaniensis LGP32, V. neptunius 09/123 1T2,* and *V. europaeus* 07/*118 T2* with some exceptions for the juvenile stage. Initially, all those pathogens were tested through

an immersion of larvae containing 1 x 10^5 cultured bacteria ml^{-1}. Immersion of spat was realized using contaminated seawater containing 2-5 x 10^5 of freshly shed bacteria, as previously described in Parizadeh et al. (2018).

Next, all families and wild stocks were also challenged at the juvenile stage by intramuscular injection with the different bacteria plus *V. chagasii 8T1-3* (Table 3), with only 2 replicates per family and wild stock (Table 3). For each pathogen except for *V. aestuarianus 02/041*, oysters were directly injected with 50 µl of a bacterial suspension into the adductor muscle, and mortality was recorded daily until day 3 post-injection. Dead oysters were removed daily. For *V. aestuarianus,* injection can lead to high mortality regardless of the level of selection for OsHV-1 resistance in *C. gigas* (Azéma et al., 2015). Thus, families and wild controls were tested using an experimental infection by cohabitation with naïve oysters injected with *V. aestuarianus*. After intramuscular injection of donor oysters, as described above, the injected oysters were transferred into 10-L tanks for 24 h. In the second step, a ratio of 3 g of injected oysters (with the shell) per 3 L of sea water was used. Thus, the injected oysters were placed for 48 h in contact with the one family or control using two 5 liters tanks filled with 3 liters of filtered and UV-treated seawater and maintained at 21 °C with adequate aeration and without adding food. Mortality was recorded daily until day 13 post-infection, the moribund oysters being removed daily. Finally, and similarly to *V. aestuarianus*, and in addition to the evaluation throughout injection, cohabitation was also done for *V. coralliilyticus 06/210,* as well as for *V. europaeus* 07/118 T2, and mortality was recorded daily for seven days.

2.6. Mortality data analyses

All statistics were performed using SAS® 9.4 software (Cary, NC, USA). Mortality was analyzed at endpoint, i.e., at day 7 for trials at the larval and spat stages, while at the juvenile stage, it was at day 7 for OsHV-1, day 13 for *V. aestuarianus* and day 3 for the other bacteria which were injected. Mortality was analyzed by a binomial logistic regression throughout the GLIMMIX procedure.

Due to some mortality for the control in the larval challenges, a first approach tested difference in mortality of *C. gigas* between the control and each of the pathogens for each trial. A second approach tested difference in mortality of *C. gigas* among pathogens within stage and trial.

Then, as OsHV-1 was tested using a bath infection protocol from larvae to juvenile, a first model tested the fixed factor stage (larvae, spat, and juvenile) at day 7. Then, mortality was analyzed within stage. For the larval and spat stages, two trials were used per stage. The models tested as fixed factors: trial (Larval 1 and 2 or Spat 1 and 2), oyster groups (families F1 to F9 and the three wild stocks), and their interactions, as well as the random factor replicates nested within trial and oyster group:

$$\text{logit}(\pi_{ijk}) = \mu + \text{trial}_i + \text{oyster group}_j + (\text{trial}_i \times \text{oyster group})_{ij} + \text{replicate}_{k(ij)}$$

where π_{ijk} is the probability of the mortality at day 7 for oyster of the *i*th trial (Larval 1 vs Larval 2 or Spat 1 vs Spat 2) for the *j*th oyster group (families F1 to F9 and the three wild stocks) at the *k*th replicate (1 to 3 for larvae and 1 to 6 for spat), and μ the intercept.

At the juvenile stage, all factors including trial were removed from the analyses.

For bacteria, mortality was analyzed among oyster groups within trial, but only for bacterial strain inducing significantly higher mortality than control.

Finally, correlation of the mortality at endpoint was calculated between OsHV-1 or *V. aestuarianus* at the juvenile stage and each of the other pathogens tested within trial and within stage, to determine if selection applied for the families could have an impact on the resistance or susceptibility to other pathogens tested in this study.

3. Results

3.1. Controls

Controls showed no mortality at the spat and juvenile stages. At the larval stage, the mean mortality was 11% and 5% for the first and second trials, respectively (Fig. 1). In detail, all families had low mortality for the first larval trial, ranging from 0 to 28%, while no mortality was observed for wild stocks (Table 4). For the second larval trial, mortality was only observed for the F2 family (59%) and the F8 family (2%) (Table 4).

3.2. Comparison of mortality of *C. gigas* among pathogens in larvae, spat and juveniles

For the first larval trial, mean mortality among oyster groups was significantly different among pathogens ($P<0.0001$) with moderate mortality for OsHV-1 (47%), intermediate for *V. neptunius* (28%), *V. coralliilyticus* (26%), *V. europaeus* (25%), *V. crassostreae* (20%), *V. tasmaniensis* (18%), *V. harveyi* (14%), and low mortality for larvae exposed to *V. aestuarianus* (1%). Larvae exposed to *V. harveyi* had similar mortality (14%) to

controls (P=0.06), those exposed to *V. aestuarianus* had significant lower mortality (1%) than controls (P<0.0001), while the other pathogens induced higher mortality than controls (P<0.0001).

For the second larval trial, significant differences in mortality among pathogens were also found, with the highest mortality for larvae exposed to OsHV-1 (71%), intermediate for *V. coralliilyticus* (44%), and the lowest for the other pathogens (<7%) (P<0.0001). Again, mortality of larvae exposed to *V. aestuarianus* was significantly lower (3%) than observed for controls (P=0.04), while those exposed to OsHV-1 or *V. coralliilyticus* had significant higher mortality than controls (P<0.0001). No difference in mortality from the control was observed for *V. crassostreae* (7%), *V. tasmaniensis* (5%), *V.neptunius* (4%), *V. europaeus (3%),* and *V. harveyi* (4%) (P>0.05).

For spat, no mortality of *C. gigas* was reported for any bacteria challenge using a bath method. Only OsHV-1 was able to induce mortality for both trials, 67% and 68% respectively.

At the juvenile stage, all pathogens induced mortality with high variation among those tested by injection. The mean mortality among oyster groups was 10% for *V. chagasii,* 18% for *V. harveyi,* 26% for *V. crassostreae,* 44% for *V. tasmaniensis,* 80% for *V. neptunius,* 88% for *V. europaeus* and 94% for *V. coralliilyticus* (Fig. 2). Moderate mortality was observed for OsHV-1 using bath (50%) and *V. aestuarianus* using cohabitation (44%) (Fig. 2). Mortality was significantly different among pathogens (P<0.0001). Finally, no mortality was observed for the cohabitation methods using *V. coralliilyticus* and *V. europaeus*.

3.3. Comparison among oyster groups for experimental infections by OsHV-1

Mean mortality among groups and trials was not significantly different between the larval (59%) and spat (68%) stages (P=0.93), while it was significantly lower at the juvenile stage (50%) (P<0.05). Mean mortality between trials or replicates per oyster group and per stage are reported in Fig. 3.

For the larval stage, interactions between the trial and the oyster groups were significant (P<0.01). Mean mortality among groups in trial 1 (47%) was significant lower than in trial 2 (71%). Nevertheless, for each trial, a significant difference in mortality was found among groups (P<0.001), with the lowest mortality for the F7 family (14%), and the highest for the F6 family (89%) (Fig. 3).

In contrast to the larval experiment, the interaction factor was not significant (P=0.32) at the spat stage as well as the trial factor (P=0.85), with similar mean mortality among groups in trials 1 (67%) and 2 (68%). Again, mortality was significantly different among groups (P < 0.0001) with the lowest mortality for the F7 family (15%), and the highest for the F3 and F4 families (97%) (Fig. 3).

At the juvenile stage, mortality was significantly different among groups ranging from 3% for the families F7 and F9 to 93% for the families F3 and F4 (Fig. 3).

3.4. Comparison among oyster groups for experimental infections by bacteria

For the larval trials showing differences in mortality between the infected and the control conditions, mortality of each group is reported on Fig. 4. Significant differences in mortality among groups were observed for *V. coralliilyticus* in both trials, ranging from 1% for wild stock 1 to 80% for the family F2 in trial 1, and from 2% for the family F1 to 100% for wild stock 2 in trial 2 (Fig.4). Significant variation in mortality among oyster groups was also reported in trial 1 for *V. crassostreae* (from 1-87%), *V. tasmaniensis* (0-69%), *V. neptunius* (2-68%), and *V. europaeus* (0-74%) (Fig. 4) ($P<0.05$).

For juveniles, mortality varied among oyster groups when exposed to each pathogenic bacterial strain tested, ranging from 85-100% for *V. coralliilyticus,* 5-70% for *V. crassostreae,* 0-75% for *V. tasmaniensis,* 60-95% for *V.neptunius,* 65-100% for *V. europaeus,* 0-40% for *V. harveyi,* 0-30% for *V.chagasi,* and 0-80% for *V. aestuarianus* (Fig. 5). Mortality was only significantly different among oyster groups for *V. crassostreae* ($P=0.02$) and *V. aestuarianus* ($P<0.05$).

3.5. Correlation of the mortality at endpoint between OsHV-1 or *V. aestuarianus* at the juvenile stage and each of the other pathogens

High and significant positive correlations were found between mortality due to OsHV-1 at the juvenile stage and the mortality due to OsHV-1 in trials Spat 1 ($r = 0.91$ and $P < 0.01$) and in Spat 2 ($r = 0.81$ and $P < 0.01$)(Table 5). Similarly, correlation was significant between mortality due to OsHV-1 at the juvenile stage and mortality of juveniles when exposed to *V. tasmaniensis* ($r = 0.70$ and $P < 0.05$). The other correlations between the mortality due to OsHV-1 in juvenile and the other pathogens tested from larvae to juvenile were not significant, nor was mortality observed in juveniles due to *V. aestuarianus* found to correlate significantly with any other mortality recorded across all experimental infections.

4. Discussion

Mortality related to OsHV-1 among oyster families/groups has been broadly investigated worldwide in spat to adult *C. gigas* (Azéma et al., 2017b; Burge et al., 2006; Dégremont et al., 2010; Divilov et al., 2019; Gutierrez et al., 2020; Gutierrez et al., 2018; Hick et al., 2018; Prado-Alvarez et al., 2016). A significant genetic basis for OsHV-1 resistance was demonstrated and a breeding program could enhance survival of spat as demonstrated by Dégremont et al. (2015b). Nevertheless, only one study deeply investigated the correlation in mortality of *C. gigas* families among spat, juveniles and adults (Azéma et al., 2017b). Concerning larvae, this stage is highly susceptible to OsHV-1 but most studies have used unselected oysters (Burge and Friedman, 2012; Le Deuff et al., 1994; Nagai and Nakamori, 2018). It would be of great importance to optimize breeding programs by testing oyster families as soon as the larval stage, in order to reduce the cost of the genetic evaluation. To our knowledge, only two studies investigated the effect of selection to enhance resistance to OsHV-1 at the larval stage. The first showed that parental origin did not have an effect on larvae mortality levels while it did at the spat stage (Prado-Alvarez et al., 2015). The second only used one resistant family and one control, and showed a difference in mortality between the two groups (Dégremont et al., 2016). Although mortality due to OsHV-1 was highly correlated between the spat and juvenile stages (Table 5), our study reveals that two of the resistant oyster families (F8 and F9) to OsHV-1infection at the juvenile stage had high mortality at the larval stage (Table 1) (Fig. 3) (Supplementary table 1). Only the F7 family showed high resistance to OsHV-1 at the larval stage in our conditions (6 well plate, 7 days, concentration 10^4 OsHV-1 DNA copies μL^{-1}). This family had the highest

breeding value for OsHV-1 resistance at the spat and juvenile stages according to Azéma et al. (2017b), indicating that it could be possible to identify only the families with the highest potential of resistance to the infection by OsHV-1 as soon as the larval stage. The F7 family might be able to activate the antiviral response a few days after fertilization while it takes longer for other resistant families or that the viral dose was too high. Further experiments should be conducted to test the resistance of *C. gigas* larvae over several dilutions using the bath method to figure out the best dose to assess the relationship between the mortality at the larval stage and the mortality at the spat stage. The larval experiments developed in this study do represent a powerful approach for early detection of the best family and larval trials would be of a great interest for breeding programs that focus on OsHV-1 resistance.

Lower mortality in Larval 1 than in Larval 2, the interaction between trials and families at this stage, and some mortality observed for the controls revealed the importance to replicate experiments at this stage to certify the resistance to OsHV-1 of each oyster family/group tested, without performing another experimental infection at the spat stage. Indeed, only families showing high resistance in all trials during the larval stage should be selected for the breeding program, such as the F7 family, while family F3, which showed low mortality in Larval 1, had high mortality due to OsHV-1 for all other trials from larvaL to juvenile stages (Supplementary table 1). This difference might be in relation to the growth potential of the larvae that increased in older larvae by culling non-viable larvae and reducing the larval density in the larval tank before sampling them for the trial 2.

As concerns *V. aestuarianus*, the bath methods used at the larval and spat stages failed to induce mortality at day 7 post-infection, while experimental infection by cohabitation managed to produce differences in mortality among families. As the susceptibility to *V. aestuarianus* increased with age and size (Azéma et al., 2016; Azéma et al., 2017b), it seems that testing *C. gigas* smaller than 5 mm in bath method is irrelevant. Similarly, bath method failed to induce a single dead oyster for both trials at the spat stage for all other bacteria strains. We hypothesize that our experimental conditions were not well suited to the questions (dose, containers used, duration of the experiment) or that *C. gigas* spat are not susceptible to these pathogens at a size of 5 mm. While cohabitation works for *V. aestuarianus* in juveniles, this method failed with *V. coralliilyticus* and *V. europaeus*. We can rule out a lack of susceptibility of juveniles to these pathogens, as high mortality was observed throughout injection (Fig. 2). Consequently, it is important to identify the best infection method to evaluate the susceptibility of oyster families, which should be adapted to the size of the oyster. Indeed, it will be challenging and time consuming to inject small spat, and impossible for larvae which are too small and fragile.

Beside OsHV-1, it appears that *V. coralliilyticus* induced the highest mortality in *C. gigas* larvae and juveniles among all bacteria tested (Fig.1-2). Whereas differences in mortality were evidenced among families and wild controls in both larval trials (Fig. 4), mortality was high (>80%) for all families at the juvenile stage (Fig. 5). Our study is the first to report (1) mortality caused by this bacterium in *C. gigas* juveniles and (2) variation among oyster groups in larvae, as previous experiments only test one batch of larvae and showed either high mortality (Richards et al., 2015), intermediate mortality (Kesarcodi-Watson et al., 2012) low mortality (Genard et al., 2013), or all three in a dose-dependent manner (Kim et al., 2019). In contrast, high variation in mortality was observed at the Larval 1 and juvenile trials for *V. crassostreae, V. tasmaniensis, V. neptunius,*

and *V. europaeus,* and in a lesser extent for *V. harveyi, V. chagasi* (Fig. 4-5). Nevertheless, none of this mortality correlated to the two pathogens for which oysters were selected for (OsHV-1 or *V. aestuarianus*), except between *V. tasmaniensis* at the juvenile stage (Table 5). This bacterium was first isolated from oysters of the Marennes-Oléron Bay in 2001 and it is naturally present in the area where wild broodstocks were sampled to produce the families/wild controls, and where is located our hatchery and our facilities dedicated to the experimental infections. It could be supposed that resistance to this bacteria was co-selected for along with OsHV-1 at the juvenile stage.

5. Conclusions

In summary, breeding programs focusing on dual resistance against OsHV-1 and *V. aestuarianus* could screen oyster families with very high resistance to the virus as soon as the larval stage by a bath method, and then select those showing the highest resistance to the bacteria using the cohabitation method at the juvenile stage. Nevertheless, such a strategy will exclude all families that develop resistance to the virus at older stages. Selection for disease resistance to OsHV-1 and *V. aestuarianus* seems not to negatively or positively impact the susceptibility to other major pathogens involved in mortality outbreaks in hatchery, nursery, or field settings (*V. coralliilyticus, V. crassostreae, V. tasmaniensis, V. neptunius, V. europaeus, V. harveyi, V. chagasii)*. Finally, our study showed that larvae had the highest mortality when exposed to OsHV-1, followed by *V. coralliilyticus* for a bath method. Unfortunately, this experimental infection method was not adapted for all bacteria strains for spat (5 mm). Beside OsHV-1 and *V. aestuarianus*, high mortality was induced in juveniles by injecting *V. coralliilyticus*, *V. europaeus,* or *V. neptunius*. Variation in

mortality observed among families/wild controls for these pathogens suggest differences in susceptibility among oysters, which could be a first step for breeding programs focusing on multiple pathogens in *C. gigas*.

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/* Due to Endnote scripts, this will deleted in the final version, because the two followings references appears only in Table 3 (Garnier et al., 2007;

Lemire et al., 2015)*/

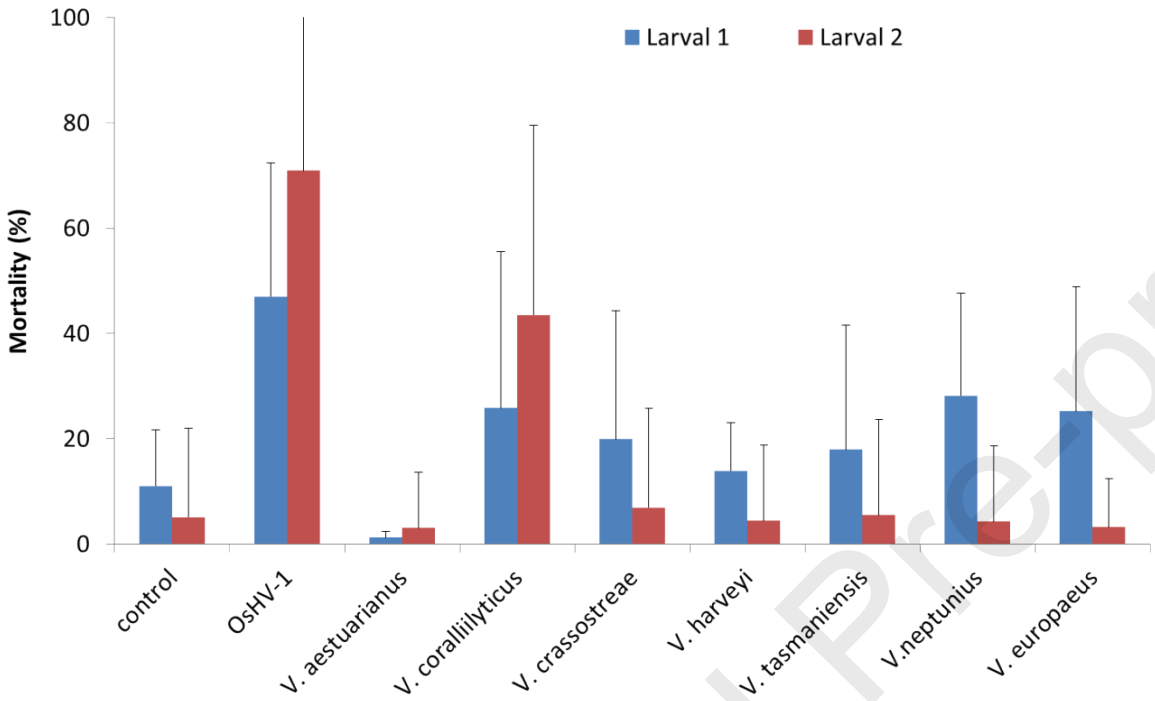


Fig. 1 Mean mortality (% + SD among oyster families and wild stocks) for the control and each pathogen using bath method in *C. gigas* larvae.

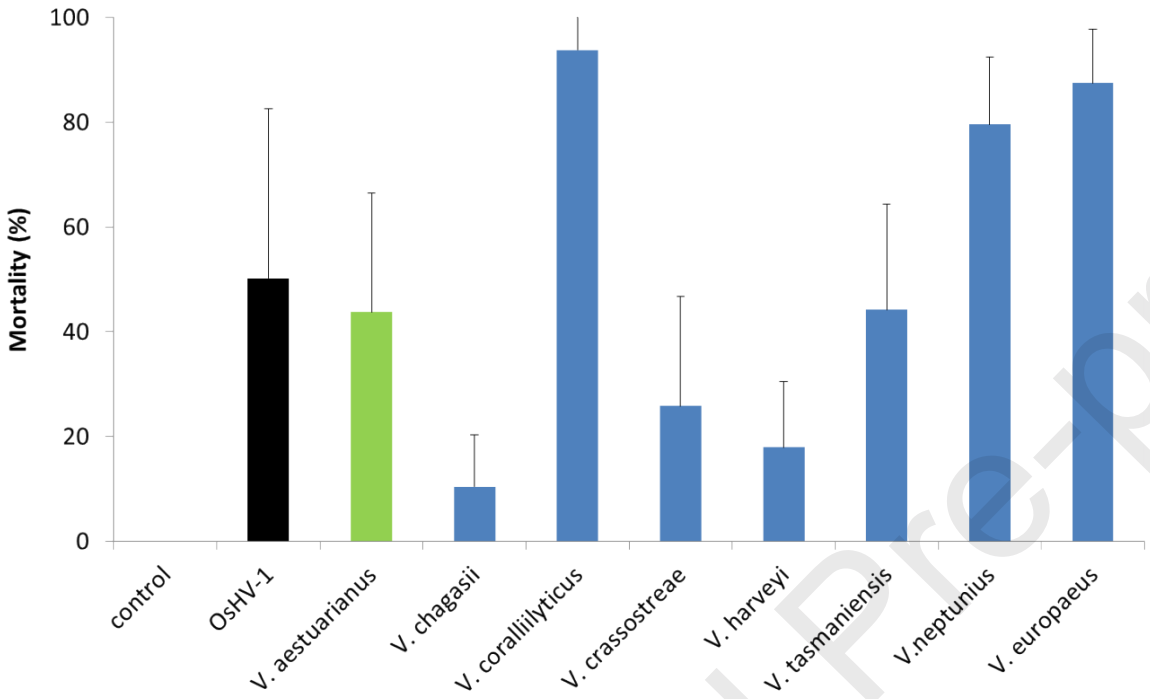


Fig. 2 Mean mortality (% + SD among oyster families and wild stocks) for the control and each pathogen using bath method (in black), cohabitation method (in green), or injection (in blue) in *C. gigas* juvenile.

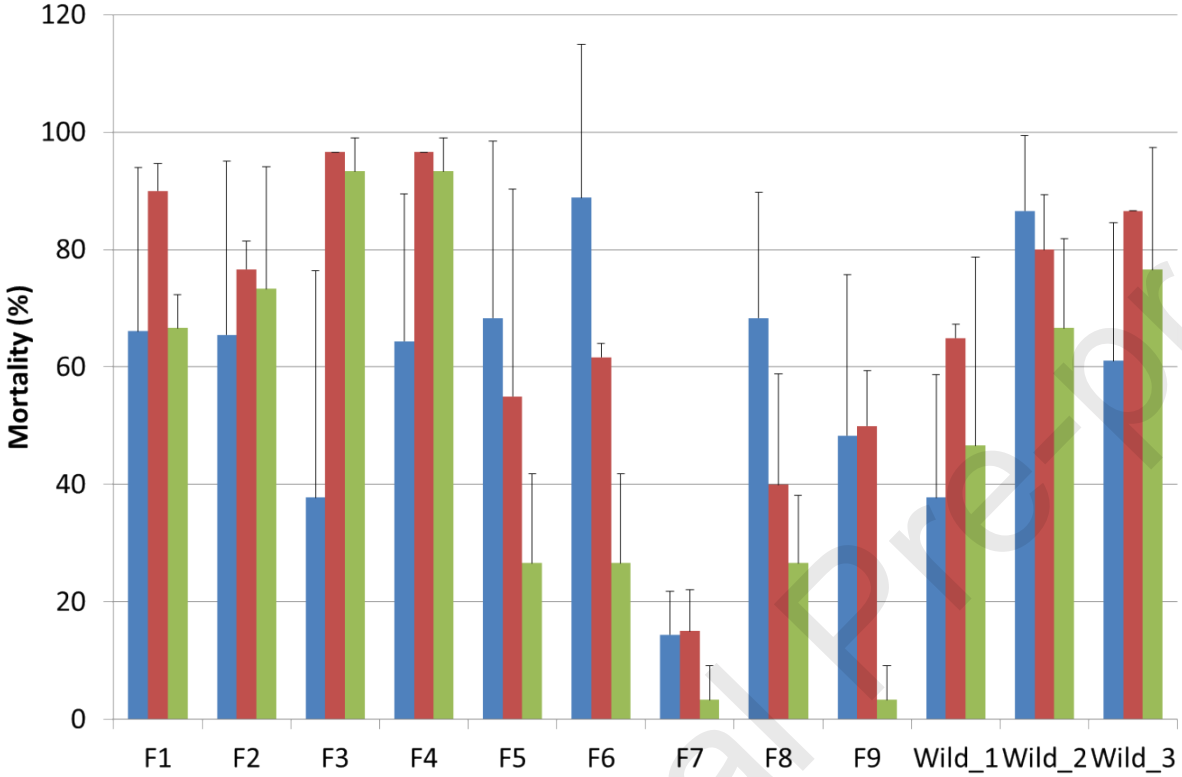
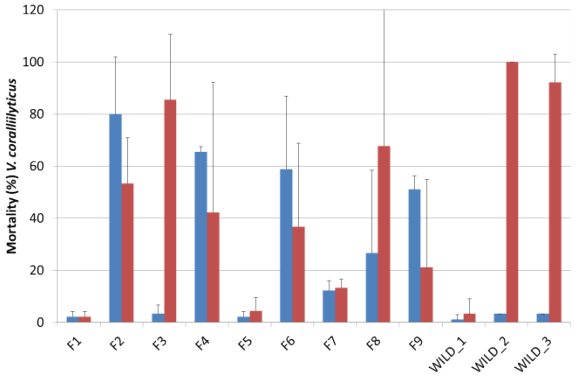
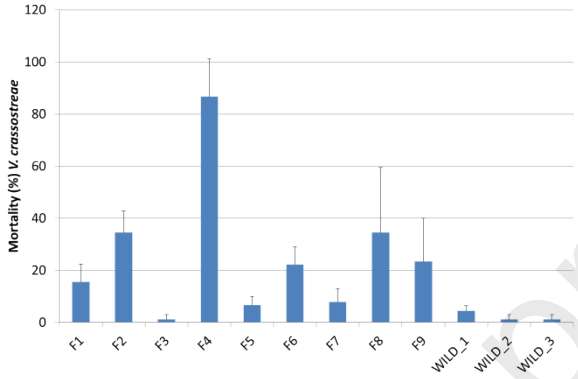
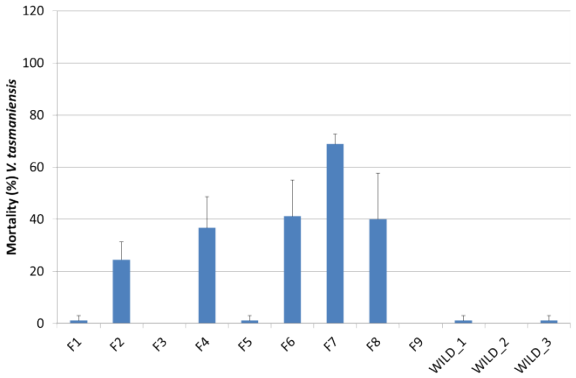
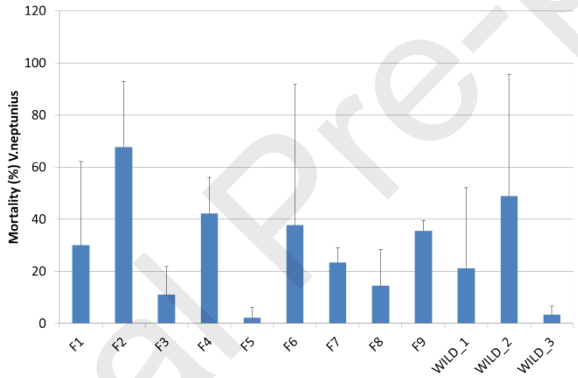


Figure 3: Mortality (% + SD among trials within stage for the larval and spat trials, and among replicates for the juvenile stage) 7 days post-infection by OsHV-1 (bath method) for the nine families and the three wild stocks at the larval (in blue), spat (in red) and juvenile (in green) stages.









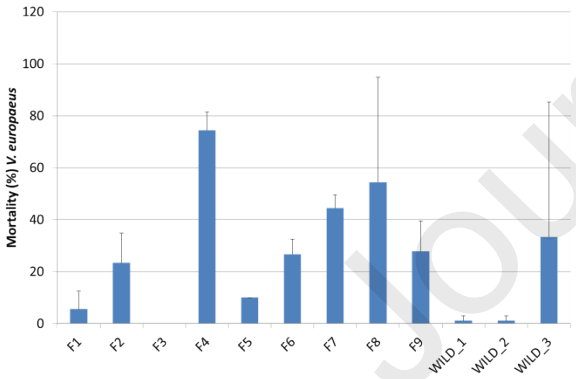
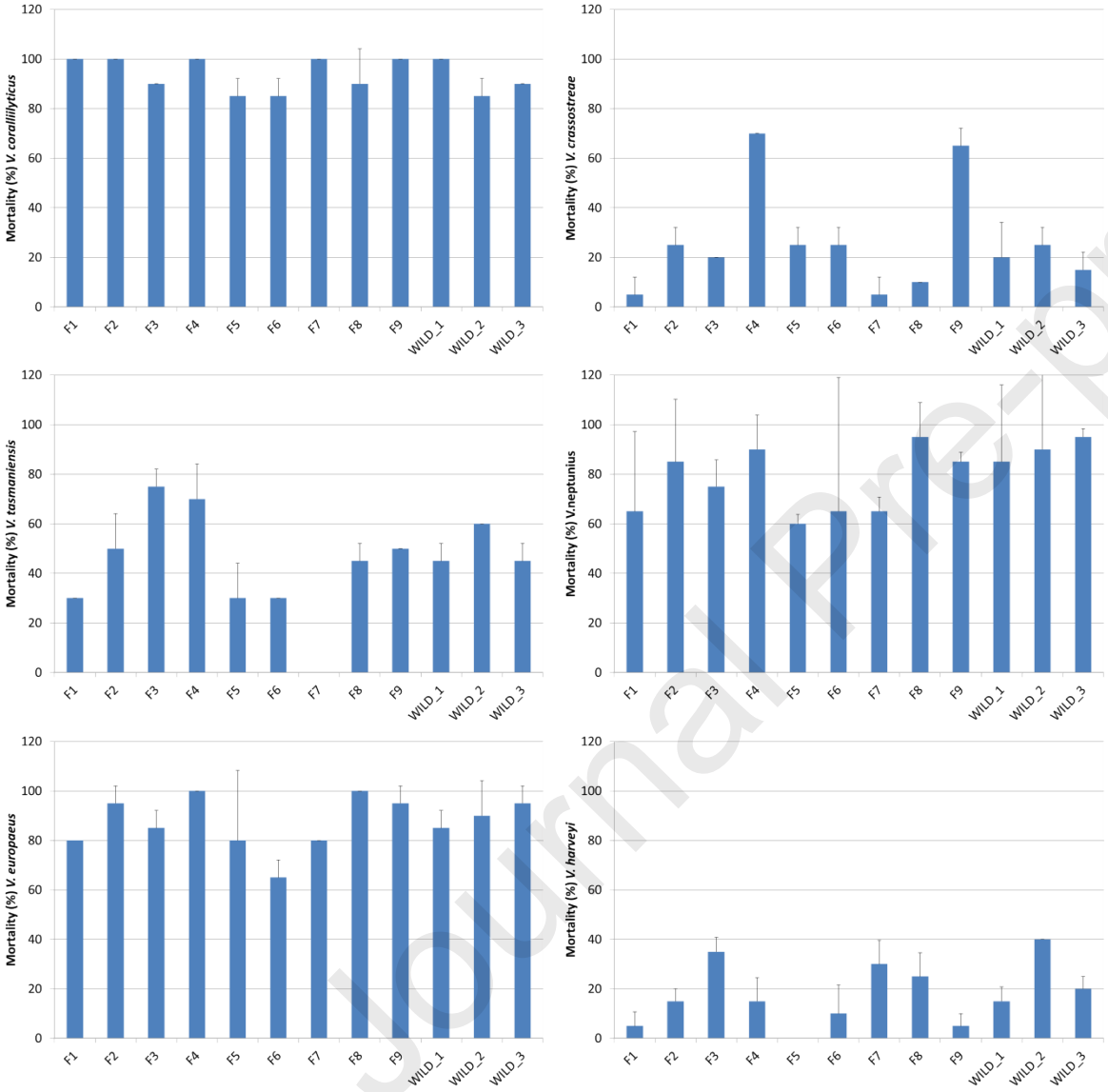


Figure 4: Mortality (% + SD among replicates) 7 days post-infection by *V. coralliilyticus, V. crassostreae, V. tasmaniensis, V.neptunius,* and *V. europaeus* for the nine families and the three wild stocks in Larval 1 (blue) and Larval 2 (in red) using a batch method. Due to the absence of difference in mortality between the control and experimental infection using bacteria, data are not showed for *V. harveyi* in both larval trials, and for Larval 2 except for *V. coralliilyticus.*



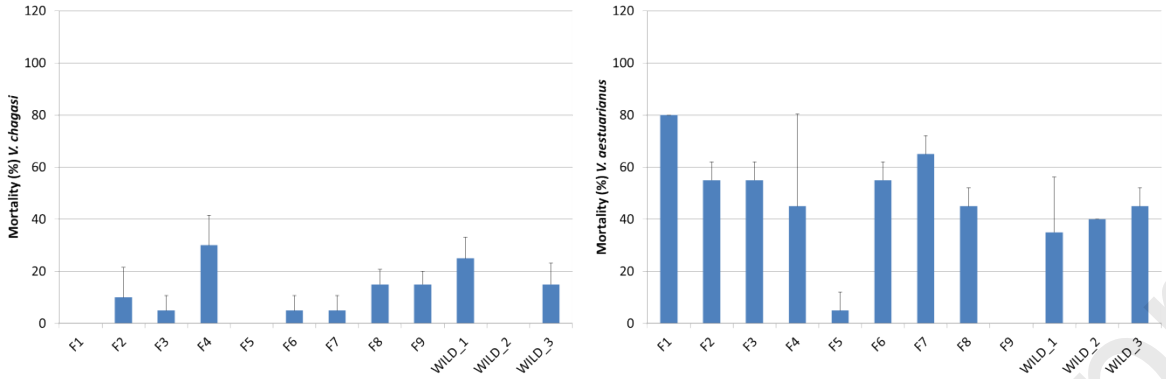


Figure 3: Mortality (% + SD among replicates) 3 days post-injection by *V. coralliilyticus, V. crassostreae, V. tasmaniensis, V.neptunius, V. europaeus, V. harveyi, V. chagasi, and 13 days post-infection by V. aestuarianus* (cohabitation) for the nine families and the three wild stocks at the juvenile stage.

Table 1: Oyster families or origins

Broodstock[1]	Generation[2]	Number of parents	Inbreeding rate F	*Susceptibility to*[3]	
				OsHV-1	*Vibrio aestuarianus*
F1	G1	17	0.25	Susceptible	Susceptible
F2	G1	20	0.25	Susceptible	Susceptible
F3	G1	18	0.25	Susceptible	Moderate
F4	G1	18	0.25	Susceptible	Moderate
F5	G1	10	0.25	Susceptible	Resistant
F6	G1	12	0.25	Moderate	Moderate
F7	G1	9	0.25	Resistant	Susceptible
F8	G1	12	0.25	Resistant	Moderate
F9	G1	18	0.25	Resistant	Resistant
Wild-1	G0	30	0	unknown	unknown
Wild-2	G0	11	0	unknown	unknown
Wild-3	G0	20	0	unknown	unknown

[1] Each G0 family (F1 to F9) was initially produced by mating one female and one male
[2] G for generation
[3] from experimental infection occurring at the juvenile stage

Table 2: List of bacterial strains used

Species	Strain	Sample origin	Sampling location	Sampling date	Identification
V. aestuarianus	02-041	Diseased cupped oysters	France	2002	Garnier et al. 2007
V. chagasii	8T1_3	Diseased cupped oysters	France	2014	Bruto et al. 2017
V. crassostreae	J2-9	Diseased cupped oysters	France	2013	Lemire et al. 2015
V. coralliilyticus	06-210	Diseased cupped oysters	France	2006	Travers et al. 2014
V. europaeus	07-118_T1	Diseased cupped oysters	France	2007	Travers et al. 2014
V. harveyi	08/076_3T1	Diseased cupped oysters	France	2008	Saulnier et al. 2010
V. neptunius	09-123_1T2	Diseased cupped oysters	France	2009	This publication
V. tasmaniensis	LGP32	Diseased cupped oysters	France	2001	Gay et al. 2004

Table 3: Experimental infections conducted for all families and wild stocks

Date	03/30/16	04/01/16	05/26/16	06/02/16	12/16 and 01/17
Stage and trial number	Larval 1	Larval 2	Spat 1	Spat 2	Juvenile[1]
Size	100 µm	150 µm	5 mm	5mm	20g ~ 50-60mm
OsHV-1	bath	bath	bath	bath	bath
V. aestuarianus 02/041	bath	bath	bath	bath	cohabitation
V. coralliilyticus 06/210	bath	bath	bath	bath	injection and cohabitation
V. crassostreae J2-9	bath	bath	bath	bath	injection
V. harveyi 08/076_3T1	bath	bath	bath	bath	injection
V. tasmaniensis LGP32	bath	bath	bath	bath	injection
V.neptunius 09/123 1T2	bath	bath	bath	bath	injection
V.europaeus 07/118 T2	bath	bath	bath	bath	injection and cohabitation
V. chagasii 8T1-3					injection
Container	6-well plate	6-well plate	6-well plate	6-well plate	5l tank
Number of replicates[2]	3	3	6	6	3 or 2
Number of individual per replicate	100[3]	100[3]	5	5	10

[1] 3 replicates for OsHV-1, and 2 for the other pathogens

[2] The number of replicates is given per family or wild stock and per pathogen

[3] This number could vary +/-30%, as it cannot be possible to put the exact number of larvae due to their small size. Mortality was recorded from the first 30 larvae (dead+alive) observed using a binocular magnifier.

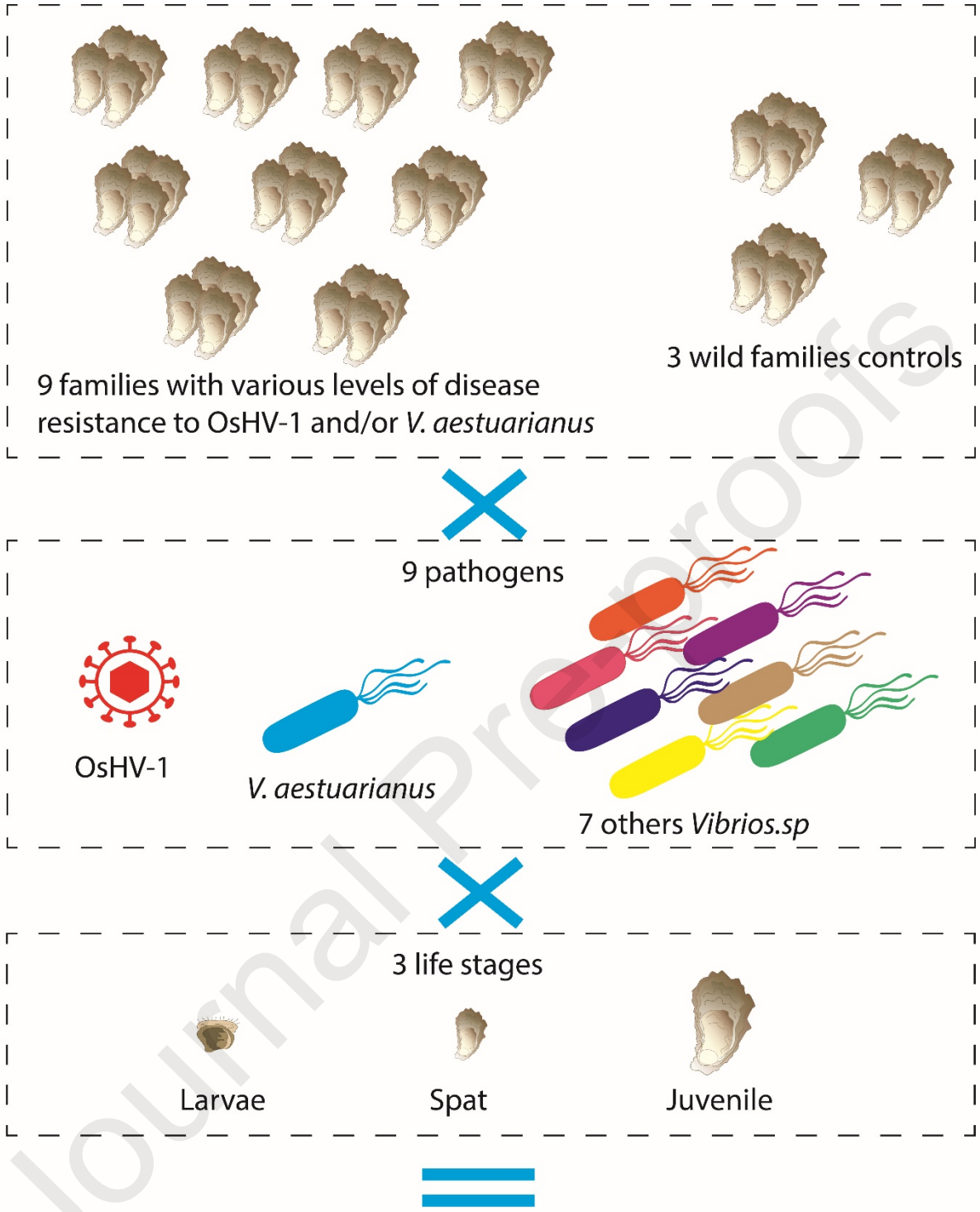
Table 4: Mortality for the controls at each experimental infection

Family/wild stock	Larval_1	Larval_2	Spat_1	Spat_2	Juvenile
F1	1.1 ± 1.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F2	27.7 ± 11.7	58.8 ± 8.9	0 ± 0	0 ± 0	0 ± 0
F3	2.2 ± 1.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F4	22.2 ± 12.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F5	3.3 ± 3.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F6	21.1 ± 3.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F7	21.1 ± 8.4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
F8	16.6 ± 8.8	2.2 ± 1.9	0 ± 0	0 ± 0	0 ± 0
F9	16.6 ± 15.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Wild_1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Wild_2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Wild_3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 5: Correlations between the mortality at endpoint by OsHV-1 or *V. aestuarianus* at the juvenile stage and each of the other pathogens (sorted by ascending P value)

	OsHV-1 juvenile			*V. aestuarianus* juvenile	
	r	P		r	P
OsHV-1_spat1	0.91	<.0001	V_crass_juv	-0.55	0.06
OsHV-1_spat2	0.81	<0.01	V_aestu_lar1	0.45	0.15
V_tasm_juv	0.70	<0.05	OsHV-1_spat1	0.43	0.17
V_cora_lar2	0.52	0.09	V_tasm_lar1	0.39	0.22
V_har_lar1	-0.43	0.16	OsHV-1_juv	0.38	0.23
V_aestu_lar1	-0.39	0.21	V_har_juv	0.34	0.28
V_aestu_juv	0.38	0.23	V_euro_juv	-0.24	0.45
V_tasm_lar1	-0.36	0.25	V_cora_juv	0.21	0.52
V_nept_juv	0.34	0.28	V_nept_lar1	0.20	0.53
V_euro_juv	0.31	0.33	V_crass_lar2	0.19	0.56
V_har_juv	0.29	0.36	V_tasm_juv	-0.18	0.57
V_euro_lar2	0.23	0.47	V_euro_lar2	0.16	0.61
V_tasm_lar2	0.22	0.48	V_har_lar2	0.16	0.62
V_aestu_lar2	0.22	0.48	V_tasm_lar2	0.16	0.63
V_nept_lar2	0.22	0.49	V_nept_juv	-0.16	0.63
V_crass_lar1	0.22	0.49	V_nept_lar2	0.16	0.63
V_har_lar2	0.21	0.50	V_aestu_lar2	0.16	0.63
V_chaga_juv	0.21	0.52	V_chaga_juv	-0.15	0.64
OsHV-1_lar2	0.19	0.55	V_cora_lar2	0.12	0.71
V_crass_lar2	0.19	0.56	OsHV-1_lar2	-0.10	0.76
V_nept_lar1	0.16	0.62	V_har_lar1	-0.08	0.82
OsHV-1_lar1	0.13	0.68	V_euro_lar1	0.05	0.87
V_euro_lar1	-0.09	0.78	V_cora_lar1	-0.04	0.91
V_crass_juv	0.08	0.81	V_crass_lar1	0.04	0.91
V_cora_juv	0.01	0.98	OsHV-1_spat2	-0.03	0.94
V_cora_lar1	-0.01	0.99	OsHV-1_lar1	0.01	0.99

V_aestu for V. aestuarianus, V_cora for V. coralliilyticus, V_crass for V. crassostreae, V_har for V. harveyi, V_tasm for V. tasmaniensis,V_nept for V.neptunius, V_euro for V. europaeus, and V_chaga for V. chagasi
lar1, lar2, spat1, spat2, and juv for larval 1, larval 2, spat1, spat 2, and juvenile trials, respectively.



Optimization of breeding programs to enhance disease resistance and its impact on other relevant pathogens in *C. gigas*

Hightlights

- From the 12 families/wild stocks tested, only one was resistant to OsHV-1 from larvae to juvenile,
- No mortality by *V. aestuarianus* using bath for oysters lesser than 5 mm,
- Breeding programs on disease resistance should now focus on *V. coralliilyticus* which induced moderate mortality in larvae and high mortality in juveniles,
- No impact of the selection for OsHV-1 and/or *V. aestuarianus* resistance to other virulent strains of *Vibrios* of *C. gigas,* except for *V. tasmaniensis*

Graphical abstract