



Resistance to OsHV-1 Infection in *Crassostrea gigas* Larvae

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The ostreid herpesvirus (OsHV-1) is one of the major diseases that affect the Pacific oyster *Crassostrea gigas*. Selective breeding programs were recently shown to improve resistance easily to OsHV-1 infections in spat, juvenile, and adult oysters. Nevertheless, this resistance has never been investigated in larvae, whereas this developmental stage has crucial importance for the production of commercial hatcheries, as well as explaining the abundance of spatfall. A first trial tested several viral suspensions at several concentrations using contaminated water with OsHV-1 in 4- and 10-day-old larvae that were produced from an unselected broodstock. In follow up on the results, one viral suspension at a final concentration of 10⁺⁶ OsHV-1 DNA copies per L was used to assess resistance to OsHV-1 infection in *C. gigas* larvae that were produced from selected and unselected broodstock. A second trial evaluated OsHV-1 resistance in larvae from both broodstocks in trials 2a, 2b, and 2c with 4, 10, and 16-day-old larvae for 7 days, which corresponded to post D larvae, umbo larvae, and eyed larvae, respectively. The mortality of unchallenged larvae for both stocks were low (<15%) at day 7 in trials 2a and 2b, whereas it ranged from 48 to 56% in trial 2c. More interestingly, selected larvae had significantly lower mortality than unselected larvae when exposed to OsHV-1 in all of the trials. Thus, the mortality was 11 and 49% for the selected larvae at day 7 post-exposure in trials 2a and 2c, respectively, in comparison with 84 and 97% for the unselected larvae. Although this difference in mortality was observed at day 5 in trial 2b, it was reduced at day 7, to 86 and 98% for the selected and unselected larvae, respectively. For the first time in the literature, the difference in mortality or the delayed onset of mortality between selected and unselected larvae have indicated a genetic resistance to OsHV-1 infection at the larval stage. Such finding should facilitate the selective breeding programs focusing on resistance to OsHV-1 infection by reducing the span of the genetic evaluation, and thus decreasing its cost.

Keywords: larvae, *Crassostrea gigas*, OsHV-1, disease resistance, mortality

INTRODUCTION

Global oyster production is largely driven by the Pacific oyster *Crassostrea gigas*, which represented 97% of production, totaling 4.7 million tons in 2012 and 4 billion US dollars (FAO, 2014). Disease resistance has been investigated in numerous oyster species, with significant responses to selection in improving resistance to protozoan, bacteria and viruses such as *Haplosporidium nelsoni* and

Perkinsus marinus in *Crassostrea virginica* (Ford and Haskin, 1987; Ragone Calvo et al., 2003), *Marteilia sydneyi* and *Bonamia roughleyi* in *Saccostrea glomerata* (Dove et al., 2013), *Bonamia ostreae* in *Ostrea edulis* (Naciri-Graven et al., 1998), *Roseovarius crassostrea* in *C. virginica* (Barber et al., 1998), and Ostreid herpesvirus type 1 (OsHV-1) in *C. gigas* (Dégremont et al., 2015c). Most of these studies have focused on the spat and adult stages, which could be explained by the cultivation of these stages by oyster farmers (Dégremont et al., 2015a). However, disease resistance is poorly investigated in larvae, although disease could have a significant impact on spatfall in the wild. Similarly, hatchery production has increased tremendously in recent times, in particular in *C. gigas* in France, and larval mortality related to disease remains a threat to oyster farmers in terms of the regularity of spat production throughout the whole year.

Pathogens reportedly induce larval mortality in several oyster species, such as *Vibrio coralliilyticus*, *V. tubiashii*, or *Vibrio* spp in *C. gigas* and *C. virginica* (Estes et al., 2004; Mersni-Achour et al., 2015; Richards et al., 2015) or *V. neptunius* and *V. ostreicida* in *O. edulis* (Prado et al., 2005, 2014). With respect to the ostreid herpes virus, OsHV-1 has been detected since 1991 during mortality-inducing outbreaks in *C. gigas* larvae, which were produced in hatcheries in France (Nicolas et al., 1992) and New Zealand (Hine et al., 1992). More recently, OsHV-1 was also detected in larvae that were produced by hatcheries in South Korea, but there is no information on the health status of the larvae (Hwang et al., 2013). The experimental horizontal transmission of OsHV-1 within *C. gigas* larvae was successfully developed under controlled laboratory conditions in 2-day-old larvae (Le Deuff et al., 1994), 3-day-old larvae (Arzul et al., 2001), and 7-day-old larvae (Burge and Friedman, 2012). Nevertheless, all these studies employed larvae that were produced from unselected broodstock.

In France, higher mortality is now routinely reported in *C. gigas* spat and juveniles in relation to OsHV-1, which has been the case since 2008 (EFSA, 2010, 2015). A specific genotype called OsHV-1 μ Var (GenBank accession no. HQ842610) was ascribed to this mortality (Segarra et al., 2010), which was also reported in New Zealand (Keeling et al., 2014), Australia (Jenkins et al., 2013), and several European countries (Peeler et al., 2012; Roque et al., 2012). Regardless of the OsHV-1 genotype, the selective breeding program was shown to easily enhance OsHV-1 resistance in *C. gigas* spat, juveniles, and adults (Dégremont et al., 2013, 2015b), and this genetic resistance is higher for older and larger animals (Dégremont, 2013; Dégremont et al., 2015c). Nevertheless, this resistance was never previously investigated in larvae.

This study describes the first investigation of resistance to OsHV-1 infection in *C. gigas* larvae from unselected broodstock and broodstock that was selected for its higher OsHV-1 resistance. Two trials were designed to track the mortality rate in larvae under experimental infection conditions. The first trial was set up to test different viral concentrations (based on the number of OsHV-1 DNA copies) with 4-day-old (trial 1a) and 10-day-old (trial 1b) unselected larvae. The second trial investigated OsHV-1 resistance in larvae with selected and unselected broodstocks. This trial was performed in 4-day-old (trial 2a), 10-day-old (trial

2b), and 16-day-old (trial 2c) larvae corresponding to D or post D veliger larvae (80–100 μ m), umbo larvae (110–200 μ m), and eyed larvae (250–350 μ m), respectively. Their mortality was recorded at days 3, 5, and 7 post-exposure, and the detection of OsHV-1 DNA was performed by real-time quantitative PCR in larvae at each date, as well as in seawater at days 0 and 7.

MATERIALS AND METHODS

Broodstocks

The first broodstock consisted of wild adult oysters that were sampled from the dock at La Tremblade in 2010, and it was used to produce the unselected larvae. The second broodstock was the eighth generation of a bi-parental family that had been produced and selected for its higher resistance to the summer mortality phenomenon in *C. gigas* in France (Dégremont et al., 2007, 2010). This family was then reproduced over eight generations from 2002 to 2010, with \sim 30 parents per generation. The family was subsequently found to have higher resistance to OsHV-1 from 2001 to 2003 (Dégremont, 2003) and to OsHV-1 μ Var in the context of the massive mortality that has been occurring in France since 2008 (Dégremont, 2011; Dégremont et al., 2016). The selected broodstock was used to produce selected larvae. Both broodstocks were held until spawning in the conditioning room of the Ifremer hatchery in La Tremblade, by maintaining the oysters in a seawater temperature at 20°C. This water was UV-treated and enriched with the alga *Skeletonema costatum*.

Spawn and Larvae

Unselected larvae were produced in April of 2011 for trials 1a and b, and unselected and selected larvae were produced in June 2011 for trials 2 a, b, and c (Table 1). For each spawn, ripe oysters were opened and tissue was sampled from the gonad; this sample was spread on a slide and used to identify the sexes of the oysters under a microscope. The numbers of parents used for each cross ranged from 6 to 14 (Table 1). For each parent, the gametes were collected by stripping the gonad, which were successively sieved to remove large (>60 μ m) and small (<20 μ m) tissue debris for the eggs and to only remove the large (>60 μ m) ones for the sperm. Within the broodstock, eggs from all the females were mixed and sperm from all the males were added. After fertilization, embryos were transferred into 150-L tanks containing filtered and UV-treated seawater at 25°C, which was changed three times per week. The larvae were fed daily with the algae *Isochrysis galbana* and *S. costatum*, and they remained in the hatchery until their evaluation during experimental infection trials. Density was adjusted from 50 larvae mL⁻¹ at early-stage larvae to three larvae mL⁻¹ for late-stage larvae, which it is commonly used by commercial hatcheries. Such densities were then used for the experimental infections.

Viral Suspensions

Viral suspensions were prepared according to Schikorski et al. (2011b). In brief, gills and mantles from OsHV-1 μ Var-infected oyster spat were dissected and pooled together in a 50 mL sterile tube. All subsequent dilutions were made with 0.22 μ m-filtered

TABLE 1 | Summary of experimental OsHV-1 infections in *C. gigas* larvae.

Trial	Broodstock	Age (days)	Size (μm)	Viral suspension	Viral concentration (OsHV-1 DNA copies L^{-1})	Number of tanks per condition
1a	Unselected ($3\varphi \times 3\sigma$)	4	80–100	Vir1, Vir 2, Vir3	10^{+7} , 10^{+8} , 10^{+9} , and 0	2
1b		10	110–200	Vir2	10^{+5} , 10^{+6} , 10^{+7} , 10^{+8} , and 0	2
2a	Unselected ($6\varphi \times 4\sigma$), selected ($10\varphi \times 4\sigma$)	4	80–100	Vir2	10^{+6} and 0	3
2b		10	110–200	Vir2	10^{+6} and 0	3
2c		16	160–320	Vir2	10^{+6} and 0	3

artificial seawater (ASW). The total mass of the tissues was weighed, and 10 volumes of $0.22\ \mu\text{m}$ 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 $0.22\ \mu\text{m}$ pore sizes under sterile conditions. Filtered tissue homogenates were stored at 4°C until use.

Trial 1: Experimental Infection Trials with OsHV-1 μVar on Unselected Larvae

Before comparing the resistance of the selected and unselected larvae under OsHV-1 infection in a second trial, a first trial was set up by using unselected larvae from wild oysters to test several viral suspensions at several concentrations. The objective of this trial was to choose one viral suspension at one concentration that will induce mortality during a span of 1 week in *C. gigas* larvae.

Trial 1a on 4 Day-Old Larvae at Sizes Ranging from 80 to $100\ \mu\text{m}$

Four-day-old larvae with sizes ranging from 80 to $100\ \mu\text{m}$ were taken from the hatchery and then transferred into a quarantine room to perform the experimental infection with OsHV-1 μvar . Three viral suspensions (Vir1, Vir2, and Vir3) were tested, each of which were tested with three final concentrations (10^{+7} , 10^{+8} , and 10^{+9} OsHV-1 DNA copies L^{-1}), which corresponded to a total of nine conditions (Table 1). For each condition, the larvae ($n = 50$ larvae mL^{-1}) were placed in two 5 L tanks in 3 L of $1.0\ \mu\text{m}$ -filtered and UV-treated seawater containing $60\ \mu\text{L}$ of Flumisol[®] at 32% (a quinolone antibiotic) and the viral suspension. According to the density and the concentration, it corresponded to $2 \times 10^{+2}$, $2 \times 10^{+3}$, and $2 \times 10^{+4}$ OsHV-1 DNA copies per larva for the final concentrations 10^{+7} , 10^{+8} , and 10^{+9} OsHV-1 DNA copies L^{-1} , respectively. Additionally, two control tanks were tested using the same protocol, but without adding the viral suspension. Each tank was aerated, and the seawater temperature was maintained at a 23°C room temperature. The larvae were not fed. After 5 days, the seawater in each tank was transferred into a 5 L beaker and then homogenized, and $260\ \text{mL}$ was sampled for the mortality estimation. This volume was then filtered through a $60\ \mu\text{m}$ screen to catch dead and live larvae, which were then transferred into a 15 mL tube. Finally, the tube was gently mixed, and its mortality counts were conducted

under a binocular magnifier using three drops of $200\ \mu\text{L}$ each, which contained the larvae. This step was included to increase the larval density and better estimate the mortality rates in a small drop. Larvae that were swimming or showing any movements (without any visible protists inside the shell) were considered alive, and the others were dead (empty shells, degradation of the tissues with brownish and undistinguishable tissues). OsHV-1 detection was performed on larvae at day 0, before their transfer in the quarantine room, and on larvae from each tank at day 5. Similarly, seawater from each tank was also screened at day 0 before adding the larvae and after adding the viral suspension as well as at day 5 at the final counting. All samples were stored at -20°C until disease screening.

Trial 1b on 10 Day-Old Larvae with Sizes Ranging from 110 to $200\ \mu\text{m}$

Similarly, from the unselected larvae that were maintained in the hatchery, 10-day-old larvae at sizes ranging from 110 to $200\ \mu\text{m}$ were placed in the quarantine room for experimental infection by OsHV-1 μvar . The same protocol was used with the following modifications. One viral suspension (Vir2) was tested at four concentrations, namely 10^{+5} , 10^{+6} , 10^{+7} , and 10^{+8} OsHV-1 DNA copies L^{-1} , corresponding to 2, 20, 200, and 2000 OsHV-1 DNA copies per larva, respectively. Mortality was estimated 5 and 7 days post-exposure, and the volume that remained after taking a $260\ \text{mL}$ sample for mortality counts was returned to the 5 L beaker at day 5. OsHV-1 DNA detection was performed on the larvae at days 0, 5, and 7 and in the water at days 0 and 7 as described in trial 1a. All samples were stored at -20°C until disease screening.

Trial 2: Experimental Infection Trials with OsHV-1 μVar on Unselected and Selected Larvae

Trial 2a on 4 Day-Old Larvae with Sizes Ranging from 80 to $100\ \mu\text{m}$

Four-day-old unselected larvae at sizes ranging from 80 to $100\ \mu\text{m}$ were transferred from the hatchery to the quarantine room for the experimental infections by OsHV-1 μvar as well as 4-day-old selected larvae. For each group of larvae, six 5 L tanks containing 3 L each of $1.0\ \mu\text{m}$ filtered and UV-treated seawater containing $60\ \mu\text{L}$ of Flumisol[®] at 32% were used. Two treatments were tested with three of the tanks that were used as control treatments, which is hereafter named the unchallenged

condition, and for the second treatment, the Vir2 viral suspension was added to the three other tanks to obtain a final concentration of 10^{+6} OsHV-1 DNA copies L^{-1} , which is hereafter named the challenged condition. Each tank was aerated, the seawater temperature was maintained at a 23°C room temperature, and the larvae were not fed. The density per tank was ~ 33 larvae mL^{-1} , corresponding to a final concentration of 30.3 OsHV-1 DNA copies per larva. The mortality was estimated by following the same protocol used in trial 1b on days 3, 5, and 7 post-exposure. OsHV-1 DNA detection was performed on larvae at days 0, 3, 5, and 7 as well as in the water at days 0 and 7 as described in trial 1a. All samples were stored at $-20^{\circ}C$ until disease screening.

Trial 2b on 10-Day-Old Larvae with Sizes Ranging from 110 to 200 μm

Similarly, 10-day-old larvae with sizes ranging from 110 to 200 μm from the same spawns used in trial 2a (but kept in the hatchery) were transferred in the quarantine room. The same protocol used in trial 2a was used, except that the density per tank was ~ 27 larvae mL^{-1} , corresponding to a final concentration of 37.0 OsHV-1 DNA copies per larva. OsHV-1 DNA detection was performed in the larvae at days 0, 3, 5, and 7 as well as in the water at days 0 and 7. All samples were stored at $-20^{\circ}C$ until disease screening.

Trial 2c on 16 Day-Old Larvae with Sizes Ranging from 250 to 350 μm

Sixteen-day-old larvae with sizes ranging from 250 to 350 μm , which came from the same spawns used in trial 2a but were kept in the hatchery, were transferred in the quarantine room. The same protocol used in trial 2a was used here, except that the density per tank was ~ 3 larvae mL^{-1} corresponding to a final concentration of 333.3 OsHV-1 DNA copies per larva. OsHV-1 DNA detection was performed on the larvae at days 0, 3, 5, and 7 as well as on the water at days 0 and 7. All samples were stored at $-20^{\circ}C$ until disease screening.

OsHV-1 DNA Quantification

Total DNA was extracted from the collected larvae as described in the mortality estimation step (see trial 1a) by using ~ 50 –200 larvae per sample. Those larvae were crushed with a piston pellet and then DNA was extracted with a QIAamp tissue mini kit according to the manufacturer's protocol as described in Schikorski et al. (2011a). Similarly, total DNA was extracted from the seawater sampled after adding the viral suspension and before adding the larvae at day 0, as well as at day 5 or 7 post-exposure, depending of the trial. For each tank, the total DNA was extracted from 100 μL of seawater. A final elution of the DNA that was extracted from larval samples was performed with 100 μL of double-distilled water and 50 μL for seawater samples. The DNA concentration was analyzed spectrophotometrically (NANODROP®). A dilution of the larval DNA samples was performed to reach a final DNA concentration of 4 ng μL^{-1} . DNA that was extracted from seawater was used without dilution. Each DNA sample was tested in duplicate qPCR tests.

The detection and quantification of OsHV-1 DNA were performed for larvae and seawater with the SYBR® green real-time PCR protocol described by Pépin et al. (2008) as adapted for use with DPFor/DPRev primers to target the OsHV-1 DNA polymerase sequence (ORF 100) (Pépin, 2013). The results were expressed as the viral DNA copy number per ng of total DNA and the viral DNA copy number per μL for larvae and seawater, respectively.

Statistical Analysis

For each count, the mortality was analyzed by binomial logistic regression throughout the Genmod procedure in SAS® 9.4 software.

In using 4-day-old unselected larvae for trial 1a, the following model was used:

$$\begin{aligned} \text{Logit}(Y_{ij}) &= \log(Y_{ij}/(1 - Y_{ij})) \\ &= \mu + \text{viral suspension } i + \text{concentration } j \\ &\quad \text{viral suspension } i \times \text{concentration } j \end{aligned} \quad (1)$$

where Y_{ij} is the probability of larval death when exposed to the i th viral suspension (Vir1, Vir2, and Vir3) at the j th concentration (10^{+7} , 10^{+8} , and 10^{+9} OsHV-1 DNA copies L^{-1}), and μ is the intercept. When a significant interaction was observed, the SLICE option was used. This approach allowed for a more powerful analysis than when the model was rerun for each effect because the degrees of freedom are not reduced (Littell et al., 2002).

For 10-day-old unselected larvae in trial 1b, the model was reduced to test the OsHV-1 concentration, and statistical analyses were performed on days 5 and 7 post-exposure.

In trial 2a, 2b, and 2c, mortality was analyzed 7 days post-exposure by using the following model:

$$\begin{aligned} \text{Logit}(Y_{ij}) &= \log(Y_{ij}/(1 - Y_{ij})) = \mu + \text{stock } i \\ &\quad + \text{treatment } j + \text{stock } i \times \text{treatment } j \end{aligned} \quad (2)$$

where Y_{ij} is the probability of larval death in the i th stock (unselected or selected larvae) for the j th treatment (unchallenged or challenged by OsHV-1), and μ is the intercept.

For trials 2a, 2b, and 2c, and only for the challenged treatment, the quantification of OsHV-1 DNA was $\log(Y + 1)$ transformed. For larvae, we analyzed the data through an ANOVA using the GLM procedure and by using the following model:

$$\text{Log}(Y + 1) = \mu + \text{stock } i + \text{sampling date } j \quad (3)$$

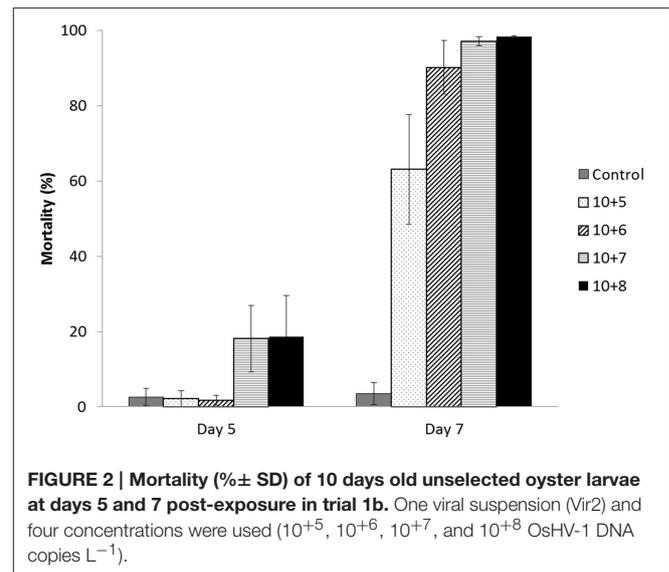
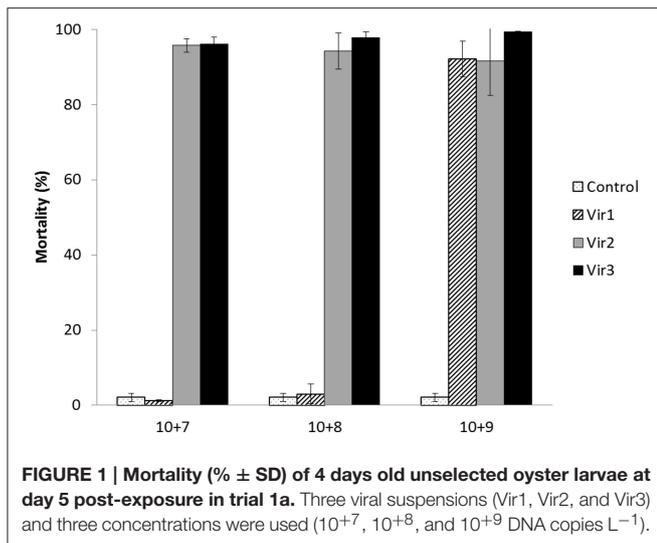
$$+ \text{stock } i \times \text{sampling date } j \quad (4)$$

where Y_i is the DNA copy number per mg of fresh tissue detected for the i th stock (unselected or selected larvae) at the j th sampling date (days 3, 5, and 7 post-exposure).

For seawater samples, data were analyzed using the following model:

$$\begin{aligned} \text{Log}(Y + 1) &= \mu + \text{stock } i + \text{sampling date } j + \text{stock } i \\ &\quad \times \text{sampling date } j \end{aligned} \quad (5)$$

where Y_{ij} is the DNA copy number per μl of seawater detected for the i th stock (unselected or selected larvae) at the j th sampling date (day 0 and day 7 post-exposure).



RESULTS

TRIAL 1: Experimental Infection Trials with OsHV-1 μ Var using Unselected Larvae

Trial 1a on 4-Day-Old Larvae with Sizes Ranging from 80 to 100 μ m

Mortality was low in unselected 4-day-old control larvae (2.1%), and ranged from 3 to 99.4% in larvae exposed to OsHV-1 at day 5 post-exposure (Figure 1). A significant interaction was found between the viral suspensions (Vir 1, Vir 2, and Vir 3) and concentrations (10⁺⁷, 10⁺⁸, and 10⁺⁹) at day 5 post exposure ($\chi^2 = 121.75$; $p < 0.0001$). For Vir 1, the larvae did not exhibit mortality at 10⁺⁷ and 10⁺⁸ OsHV-1 DNA copies L⁻¹ but they had significantly higher mortality at 10⁺⁹ OsHV-1 DNA copies L⁻¹ ($\chi^2 = 279.60$; $p < 0.0001$). For Vir 2 and 3, high mortality was observed regardless of the Vir2 concentration ($\chi^2 = 0.33$; $p = 0.85$) and Vir3 concentration ($\chi^2 = 4.93$; $p = 0.09$).

Trial 1b on 10 Day-Old Larvae with Sizes Ranging from 110 to 200 μ m

For 10-day-old unselected larvae, the mortality at day 5 post-exposure remained lower than 3% for the control and those exposed to a final concentration of 10⁺⁵ and 10⁺⁶ OsHV-1 DNA copies L⁻¹. Significantly higher mortality (18.1 and 18.6%) was found for larvae that were exposed to the two higher concentrations of OsHV-1 DNA (10⁺⁷ and 10⁺⁸; $\chi^2 = 83.68$; $p < 0.0001$; Figure 2). Two days later, the control larvae still did not exhibit mortality (<3%), and the mortality increased from 63.1% at 10⁺⁵ to 98.3% at 10⁺⁸ (Figure 2) and was significantly different among the concentrations ($\chi^2 = 119.19$; $p < 0.0001$).

Trial 2: Experimental Infection Trials by OsHV-1 μ Var using Unselected and Selected Larvae

Trial 2a on 4 Day-Old Larvae at Sizes Ranging from 80 to 100 μ m

For 4-day-old larvae at sizes ranging from 80 to 100 μ m, the mortality of the unchallenged larvae was low (<20%) at the end

of the trial on day 7 for both the unselected and selected larvae (Figure 3A). A similar mortality pattern was also observed for the selected larvae that were exposed to OsHV-1. By contrast, the unselected larvae that were challenged by OsHV-1 did not exhibit mortality on day 3, but moderate mortality was observed for one of the three tanks (36.3%) on day 5 and remained low for the two others (5.4 and 6.9%). On day 7, mortality was high (83.8%) for the unselected larvae that were challenged by OsHV-1, ranging from 63.8 to 94.3%, depending on the tank (Figure 3A).

Statistical analyses revealed a significant interaction between stocks and treatments ($\chi^2 = 22.73$; $p < 0.0001$; Table 2). At the stock level, unselected larvae had significantly higher mortality when exposed to OsHV-1 than those that were not exposed ($\chi^2 = 103.75$; $p < 0.0001$), whereas selected larvae had similar and low mortality under both conditions ($\chi^2 = 2.82$; $p < 0.0001$). At the treatment level, unselected larvae had significantly higher mortality than selected larvae under both treatments, although the level of significance was much higher under the challenge condition (Table 2).

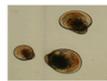
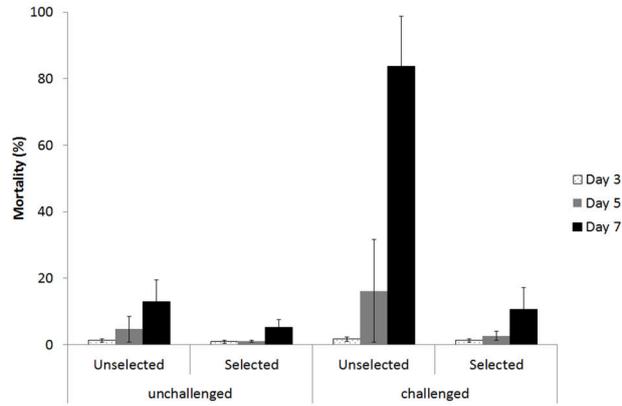
Trial 2b on 10-Day-Old Larvae with Sizes Ranging from 110 to 200 μ m

Similar to the results obtained for the unchallenged condition in trial 2a, the 10-day-old unchallenged larvae had low mortality for both stocks on day 7 (<20%) (Figure 3B). Larvae that were exposed to OsHV-1 did not exhibit mortality on day 3, and their mortality was moderate for the selected larvae (25.7%) and high for the unselected larvae (77.1%) on day 5 (Figure 3B). On day 7, the mortality was 86.4 and 97.7% for the selected and unselected larvae, respectively (Figure 3B).

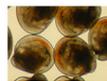
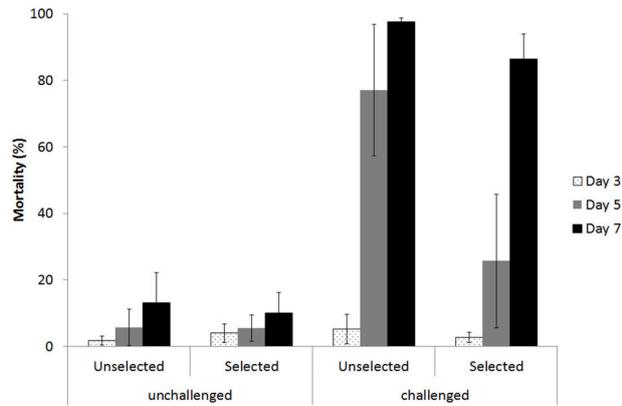
Significant interactions between the stocks and treatments were evidenced on day 7 ($\chi^2 = 11.80$; $p = 0.0006$; Table 2). At the stock level, larvae that were exposed to OsHV-1 had significantly higher mortality than those that were not exposed in both stocks on day 7 (Table 2). At the treatment level, the unselected and selected larvae had similar mortality for the unchallenged condition ($\chi^2 = 0.97$; $p = 0.32$), and the selected



A 4-day-old / 80-100 μm



B 10-day-old / 110-220 μm



C 16-day-old / 160-320 μm

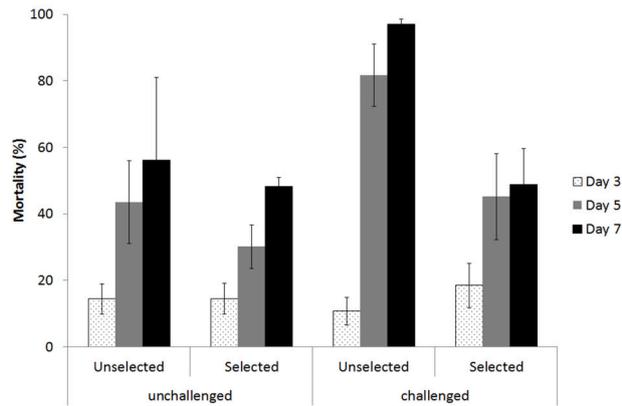


FIGURE 3 | Mortality (% \pm SD) of the unselected and selected larvae on days 3, 5, and 7 post-exposure for the unchallenged and challenged conditions (10^{+6} OshV-1 DNA copies L^{-1}) in trial 2a (A), trial 2b (B), and trial 2c (C) corresponding to 4, 10, and 17-day-old larvae, respectively and 80–100, 110–200, and 160–320 μm larvae, respectively.

TABLE 2 | Logit analysis of the mortality at the end of the trial on day 7 for unchallenged and challenged larvae when using selected OsHV-1-resistant or unselected broodstocks for 4, 10, and 16 day-old larvae in trials 2a, 2b, and 2c, respectively.

Source	df	Trial 2a		Trial 2b		Trial 2c	
		χ^2	p	χ^2	p	χ^2	p
Stock	1	101.96	<0.0001	26.65	<0.0001	26.15	<0.0001
Treatment	1	123.53	<0.0001	563.96	<0.0001	13.97	<0.0001
Stock × Treatment	1	22.73	<0.0001	11.80	0.0006	35.82	<0.0001
SLICE OPTION AT THE STOCK LEVEL^a							
Unselected	1	103.75	<0.0001	112.54	<0.0001	19.37	<0.0001
Selected	1	2.82	0.09	151.57	<0.0001	0.01	0.91
SLICE OPTION AT THE TREATMENT LEVEL^b							
Unchallenged	1	6.49	0.0109	0.97	0.32	1.42	0.23
Challenged	1	117.66	<0.0001	16.56	<0.0001	22.74	<0.0001

^aTest for each stock (selected and unselected larvae): the difference in mortality between the larvae challenged with OsHV-1 and the unchallenged larvae.

^bTest for each treatment (unchallenged and challenged larvae to OsHV-1): the difference in mortality between the selected and the unselected larvae.

larvae had significantly lower mortality than the unselected larvae when exposed to OsHV-1 on day 7 ($\chi^2 = 16.56$; $p < 0.0001$; Table 2).

Trial 2c on 16 Day-Old Larvae with Sizes Ranging from 250 to 350 μm

The 16-day-old larvae exhibited a mortality trend that regularly increased from day 3 to day 7 for both stocks under the unchallenged condition, reaching 48.2 and 56.1% for the selected and unselected larvae, respectively (Figure 3C). A similar mortality pattern was also observed for the selected larvae that were exposed to OsHV-1, with a final mortality of 48.9% on day 7. The unselected larvae experienced low mortality (10.7%) on day 3, which rapidly increased to 81.7% on day 5 and 97.1% on day 7 (Figure 3C).

Interactions between stocks and treatments in terms of mortality were again significant at day 7 ($\chi^2 = 35.82$; $p < 0.0001$; Table 2). At the stock level, selected larvae that were exposed to OsHV-1 had similar mortality to those that were unexposed ($\chi^2 = 0.01$; $p = 0.91$). In contrast, unselected larvae exposed to OsHV-1 had significantly higher mortality than those not exposed to the virus ($\chi^2 = 19.37$; $p < 0.0001$; Table 2). At the treatment level, no significant difference in mortality was found between the unselected larvae and the selected larvae under the unchallenged condition ($\chi^2 = 1.42$; $p = 0.23$). Conversely, unselected larvae that were exposed to OsHV-1 had significantly higher mortality than selected larvae ($\chi^2 = 22.74$; $p < 0.0001$; Table 2).

OsHV-1 Quantification

Before the experimental infections, OsHV-1 DNA was not detected in the larvae from all trials.

OsHV-1 Quantification in the Seawater and Larvae in Trial 1a

For 4-day-old unselected larvae in trial 1a, OsHV-1 DNA was detected in the seawater at day 0 ($<10^{+1} \mu\text{L}^{-1}$) for the control. Higher amounts were found in seawater and larvae at day 5, but they were still at a low level (10^{+3} copies per μL or ng of DNA)

TABLE 3 | Quantification of OsHV-1 DNA in seawater (copies per μL) at days 0 and 5 post-exposure, and in larvae (copies per ng of DNA) at day 5 in trial 1a.

Viral concentration (Copies per L)	Viral suspension	SeaWater		Larvae
		Day 0	Day 5	Day 5
10^{+7}	Vir1	0	$1.36 \times 10^{+4}$	$1.00 \times 10^{+2}$
10^{+7}	Vir2	1.77×10^{-1}	$1.52 \times 10^{+5}$	$5.28 \times 10^{+6}$
10^{+7}	Vir3	$1.05 \times 10^{+1}$	$7.82 \times 10^{+4}$	$4.95 \times 10^{+6}$
10^{+8}	Vir1	$3.16 \times 10^{+0}$	$2.55 \times 10^{+4}$	$9.59 \times 10^{+4}$
10^{+8}	Vir2	$1.62 \times 10^{+1}$	$1.10 \times 10^{+4}$	$3.32 \times 10^{+6}$
10^{+8}	Vir3	$2.97 \times 10^{+2}$	$8.25 \times 10^{+4}$	$4.71 \times 10^{+6}$
10^{+9}	Vir1	$1.90 \times 10^{+1}$	$9.67 \times 10^{+4}$	$3.28 \times 10^{+6}$
10^{+9}	Vir2	$2.87 \times 10^{+2}$	$1.83 \times 10^{+4}$	$3.52 \times 10^{+6}$
10^{+9}	Vir3	$1.69 \times 10^{+3}$	$8.84 \times 10^{+3}$	$2.18 \times 10^{+6}$
Control	Control	3.91×10^{-1}	$1.75 \times 10^{+3}$	$4.73 \times 10^{+3}$

for the control (Table 3). For the other conditions, OsHV-1 DNA was detected at a low level in the seawater at day 0 ($<10^{+3} \mu\text{L}^{-1}$) and at a much higher level at day 5, ranging from 10^{+4} to 10^{+5} DNA copies per μL (Table 3). Similarly, a high amount of OsHV-1 DNA was detected in the larvae at day 5 for all conditions, ranging from 10^{+5} to 10^{+7} copies per ng of total DNA except for Vir1 at the lowest concentration tested, for which it was only 10^{+2} (Table 3).

OsHV-1 Quantification in Seawater and Larvae from Trial 1b

In assessing 10-day-old larvae for trial 1b, OsHV-1 DNA was detected in the seawater at day 0 at a low level ($<10^{+2}$) and at a higher level at day 7 post-exposure, with concentrations ranging from 10^{+2} for the control to 10^{+4} for the infected conditions (Table 4). For the larvae, the amount of OsHV-1 DNA remained low in the control at days 5 and 7 ($<10^{+1}$), and it reached 10^{+6} DNA copies per ng of DNA at days 5 and 7 (Table 4).

TABLE 4 | Quantification of OsHV-1 DNA in seawater (copies per μL) at days 0 and 5 post-exposure, and in larvae (copies per ng of DNA) at days 5 and 7 in trial 1b.

Viral concentration (Copies per L)	Seawater		Larvae	
	Day 0	Day 7	Day 5	Day 7
10^{+5}	$1.44 \times 10^{+0}$	$1.26 \times 10^{+4}$	$6.47 \times 10^{+5}$	$3.71 \times 10^{+6}$
10^{+6}	$3.98 \times 10^{+0}$	$1.82 \times 10^{+3}$	$8.32 \times 10^{+5}$	$3.21 \times 10^{+6}$
10^{+7}	$2.52 \times 10^{+0}$	$5.59 \times 10^{+2}$	$7.28 \times 10^{+5}$	$2.34 \times 10^{+5}$
10^{+8}	$1.43 \times 10^{+1}$	$3.67 \times 10^{+3}$	$2.39 \times 10^{+6}$	$8.38 \times 10^{+5}$
Control	$2.53 \times 10^{+0}$	$6.60 \times 10^{+2}$	$2.05 \times 10^{+0}$	$1.43 \times 10^{+0}$

OsHV-1 Quantification in the Seawater from Trial 2abc

For 4, 10, and 16-day-old selected larvae and unselected larvae in trial 2abc, OsHV-1 was not detected in the seawater from the unchallenged condition at day 0, but it was detected for all challenged conditions after adding the viral suspension (Vir 2) containing in average 15 copies of OsHV-1 DNA per μL , and ranging from 2.94×10^{-1} to $4.38 \times 10^{+1}$ copies of OsHV-1 DNA per μL , depending of the trial (Table 5).

At day 7, similar copy numbers were observed for trial 2abc, except that a higher amount of DNA copies were detected for both stocks under the challenge condition ranging from $6.54 \times 10^{+1}$ copies DNA copies per μL for the selected larvae in trial 2c to $6.11 \times 10^{+3}$ copies per μL for the selected larvae in trial 2b (Table 5).

None of the factors (stocks, date of sampling and the interaction) was significant in trial 2a ($p > 0.05$). In trial 2b, only the date of sampling factor was found significant with higher amount of OsHV-1 DNA at day 7 post-exposure than at day 0 ($p < 0.0001$). Concerning the trial 2c, only the interaction was not significant ($p > 0.05$). Thus, the seawater sampled in tanks containing the selected larvae had a significant lower amount of OsHV-1 DNA than the seawater sampled in tanks containing the unselected larvae ($p = 0.0010$). Significant higher amount of OsHV-1 DNA was detected at day 7 than at day 0 ($p < 0.0001$).

OsHV-1 Quantification in Larvae from Trial 2abc

For 4, 10, and 16-day-old selected larvae and unselected larvae in trial 2abc, OsHV-1 DNA was detected in the larvae from the unchallenged condition, but it remained low, at 10^{+3} DNA copies per ng of total DNA, throughout the duration of all the experiments (Figures 4A–C). By contrast, a high level of OsHV-1 DNA was detected in unselected and selected larvae for the challenge condition.

Thus, in trial 2a, the OsHV-1 DNA copies per ng of total DNA ranged from 10^{+5} to 10^{+6} for both unselected and selected larvae at days 3, 5, and 7 (Figure 4A). In a closer look, the OsHV-1 amount reached a peak at day 5 post-exposure for the unselected larvae, while it was at day 7 for the selected larvae. None of the factors (stocks, date of sampling and the interaction) was significant in trial 2a ($p > 0.05$).

For trial 2b, a high amount of OsHV-1 (10^{+7}) was observed for both stocks at days 3 and 5, and it dropped significantly to 10^{+4} at day 7 ($p < 0.0001$; Figure 4B). The interaction between stocks and date of sampling was not significant ($p = 0.66$), and

the amount of OsHV-1 did not significantly differ between the selected and the unselected larvae ($p = 0.06$).

For trial 2c, the OsHV-1 amount was again high for the unselected larvae reaching a peak at day 3 ($3.31 \times 10^{+6}$), and remained at this level, although it slightly decreased at days 5 and 7 ($1 \times 10^{+6}$ and $9.32 \times 10^{+5}$, respectively). By contrast, the amount of OsHV-1 DNA increased regularly from day 3 (10^{+4}) to day 7 (10^{+5}). Interaction and date of sampling were not significant ($p > 0.05$) while the amount of OsHV-1 DNA for the unselected larvae was significantly higher than for selected larvae ($p = 0.0067$).

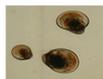
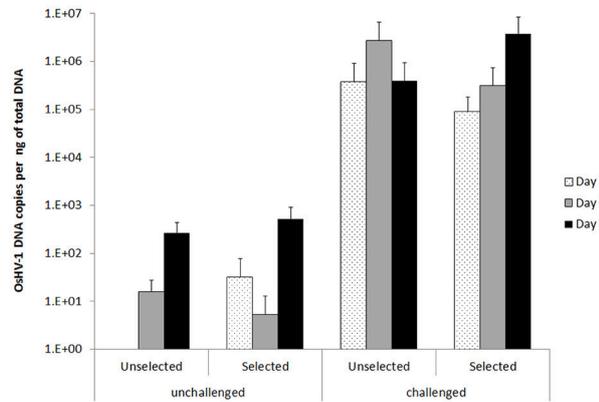
DISCUSSION

The major finding of this study revealed lower mortality from OsHV-1 in larvae through day 7 post-exposure that were produced from disease-resistant broodstock in comparison with larvae that were produced from unselected broodstock (Figures 3A–C). Consequently, OsHV-1 infection resistance in *C. gigas* could be observed at all stages, as previously demonstrated in spat, juveniles and adults (Dégremont, 2013; Dégremont et al., 2013). To our knowledge, this is the first report on a positive response to selection in oyster larvae. A previous study used an experimental challenge with bacterial pathogens in larvae from three disease-resistant lines of the eastern oysters *C. virginica* (Gomez-Leon et al., 2008). Unfortunately, the line that was selected for its higher resistance to *Roseovarius* oyster disease at the juvenile stage was not tested at the larval stage, and the two other oyster lines, which were selected for their higher resistance to *P. marinus* and/or *H. nelsoni*, were evaluated against three bacterial pathogens, namely two *Vibrio* spp strains and *Roseovarius crassostrea*. Nevertheless, the difference in mortality between lines at the larval stage was observed at 20°C , suggesting that resistance to pathogens could be present in the oyster larvae (Gomez-Leon et al., 2008). Our study strengthens their findings.

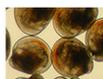
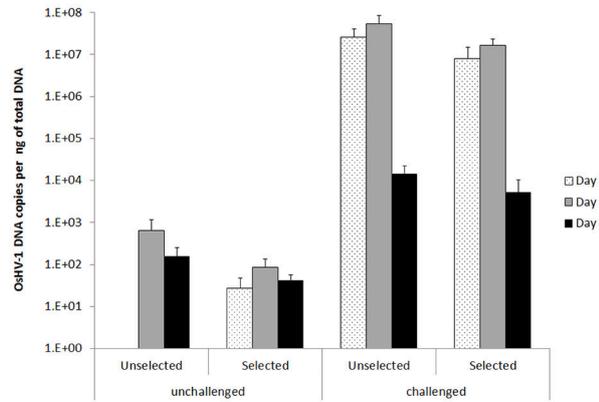
We observed high mortality in all larval stages exposed to OsHV-1 μvar from D hinge to eyed larvae. The previous studies on the horizontal transmission of OsHV-1 in larvae primarily focused on 2–3-day-old larvae (Le Deuff et al., 1994; Arzul et al., 2001) or the 7-day-old larvae without any indication about the sizes of the larvae (Burge and Friedman, 2012). At these ages, we assumed that the authors had primarily studied D larvae and small umbo larvae. Consequently, *C. gigas* larvae are susceptible to OsHV-1 during their whole planktonic existence. This result suggests that OsHV-1 is a permanent threat for hatcheries, and OsHV-1 could be an important regulator of spatfall in *C. gigas* in the open environment. Spawning usually occurs during the summer when the seawater temperature favors the OsHV-1 disease, which is commonly higher than 16°C in France (Pernet et al., 2012; Dégremont, 2013). Thus, the absence of significant *C. gigas* spatfall from 2009 to 2011 in Arcachon Bay could be explained by the high mortality caused by OsHV-1 in larvae. The pathogen hypothesis was unfortunately not investigated, but others were tested, such as the spatial distribution of broodstocks, their trophic competition, the presence of pollutants or a decrease or change in trophic resources (Bernard et al., 2014). However, some assays for OsHV-1 detection were performed on frozen



A 4-day-old / 80-100 μm



B 10-day-old / 110-220 μm



C 16-day-old / 160-320 μm

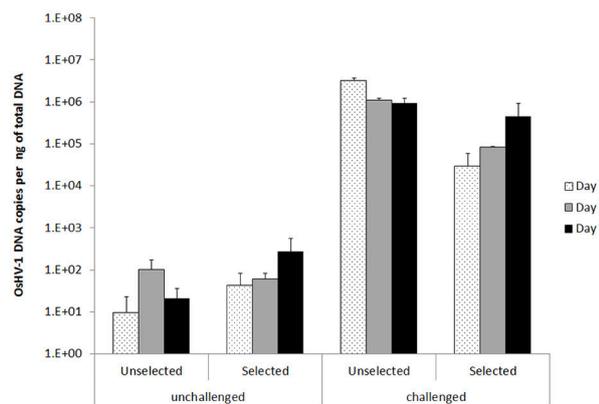


FIGURE 4 | OshV-1 DNA copies per ng of total DNA (% + SD) in unselected and selected larvae on days 3, 5, and 7 post-exposure for the unchallenged and challenged conditions (0 and 10⁺⁶ OshV-1 DNA copies L⁻¹, respectively) in trial 2a (A), trial 2b (B), and trial 2c (C) corresponding to 4, 10, and 17 day-old larvae, respectively and 80–100, 110–200, and 160–320 μm larvae, respectively.

TABLE 5 | Quantification of OsHV-1 DNA in seawater (copies per μL) for the unchallenged and challenged conditions in trial 2a, trial 2b, and trial 2c.

Trial	Age (days)	Size (μm)	Stock	Unchallenged		Challenged	
				Day 0	Day 7	Day 0	Day 7
2a	4	80–100	Unselected	Not detected	Not detected	2.94×10^{-1}	$3.16 \times 10^{+2}$
			Selected	Not detected	Not detected	$1.23 \times 10^{+1}$	$2.41 \times 10^{+2}$
2b	10	110–200	Unselected	Not detected	Not detected	$1.97 \times 10^{+1}$	$2.94 \times 10^{+3}$
			Selected	Not detected	Not detected	$4.38 \times 10^{+1}$	$6.11 \times 10^{+3}$
2c	17	160–320	Unselected	Not detected	Not detected	$1.37 \times 10^{+1}$	$4.58 \times 10^{+2}$
			Selected	Not detected	Not detected	$1.40 \times 10^{+0}$	$6.54 \times 10^{+1}$

samples from an oyster larval abundance survey in Marennes Oléron Bay. These samples were collected during the spawning season in 2009, 2010, and 2011, and they revealed a positive signal for viral DNA in the larvae (unpublished data). It would be interesting to extend studies to determine whether exposed larvae are able to settle and become spat.

For the larvae that were experimentally infected by OsHV-1, the quantity of the virus found in seawater increased significantly in all trials from days 0 to days 5 and 7 (Tables 3–5). These results suggest that OsHV-1 replicated in the larvae, which were then released in the seawater. This finding is consistent with the experimental OsHV-1 challenge in *C. gigas* spat, for which viral DNA was not detected at time 0, but it increased to 10^{+3} copies per μL by 5 days later (Schikorski et al., 2011a). In contrast to the results of Schikorski's study, OsHV-1 DNA was also detected in seawater at day 0 and days 5 or 7, and in larvae at days 5 or 7 for the unchallenged condition, but the viral copy level was low ($<10^{+3}$). Despite the presence of viral DNA, no mortality was reported in unchallenged larvae in all trials, excepted for in trial 2c, which will be explained below, suggesting that this is just a trace of viral DNA and not virulent particles. Its detection could be explained by the seawater that was pumped into the Marennes-Oléron Bay, where mortality related to OsHV-1 was reported during this period (Dégremont et al., 2016). Thus, filtration at $1.0\mu\text{m}$ and UV-treated seawater likely removed most viral particles, and the OsHV-1 DNA we detected was likely inactive viral particles or degraded DNA. This finding is consistent with a hypothesis suggesting that OsHV-1 may be carried on abioseston particulate vector of OsHV-1 from seawater using filtration at $5\mu\text{m}$ enabled *C. gigas* spat to survive in spite of the presence of OsHV-1 μVar in the water supply (Whittington et al., 2015).

For 4-day-old unselected larvae (trial 2a), the unselected and selected larvae did not suffer from mortality at day 3 (Figure 3A). In fact, the amount of OsHV-1 detected in the larvae increased from none at day 0 to 10^{+5} copies per ng of DNA at day 3 (Figure 4A), suggesting the active multiplication of the virus in both stocks. This fast proliferation in the larvae was already reported during the experimental infection of larvae by OsHV-1 (Le Deuff et al., 1994; Burge and Friedman, 2012). Although the amount of OsHV-1 was slightly higher at day 5 for both stocks, unselected larvae had 8-fold times more OsHV-1 DNA detected than selected larvae, and only unselected larvae started

dying. This is in agreement with finding obtained in OsHV-1-resistant and unselected in *C. gigas* spat tested in field condition (Dégremont, 2011). A high-mortality outbreak related to OsHV-1 was observed between days 5 and 7 post-exposure, again only for the unselected larvae. Although the OsHV-1 amount slightly decreased in unselected larvae from days 5 to 7, it was still increasing in selected larvae during this period, which still did not exhibit significant mortality (Figures 3A, 4A). Dégremont (2011) reported that the mortality peak immediately followed the viral load peak in *C. gigas* spat, suggesting that selected larvae could have experienced mortality if the trial had lasted longer. At this age, selected larvae seemed to be resistant to OsHV-1 infection, or the onset of mortality related to OsHV-1 took longer to appear, underlying the genetic resistance of *C. gigas* in small larvae.

For 10-day-old larvae (trial 2b), mortality came earlier, between days 3 and 5 post-exposure for unselected larvae, which was concomitant with the large amount of OsHV-1 DNA on both dates (Figures 3B, 4B). Though mortality was high for the unselected larvae at day 5 (77.1%), this mortality level was only observed 2 days later for the selected larvae. Thus, for the first time, we demonstrated that selected larvae can suffer high mortality from OsHV-1. This finding also revealed that the onset of mortality was delayed for the selected larvae, confirming their higher genetic resistance in comparison with unselected larvae. Even if the selected larvae experienced mortality from OsHV-1, this finding is consistent with the results found under field conditions. Indeed, resistant stocks always experienced mortality related to OsHV-1 infection in spat and juveniles, sometimes reaching 50%, whereas unselected stocks experienced higher mortality, usually reaching 90% (Dégremont, 2013; Dégremont et al., 2015c). One hypothesis to explain the higher mortality in larvae than in spat for the selected stock could be related to their younger age and smaller size. OsHV-1 resistance was shown to increase with age and size, and older animals are generally less affected (Pernet et al., 2012; Dégremont, 2013). Another hypothesis could be related to the development of the immune system. Tirape et al. (2007) showed that the immune system develops gradually throughout the life of the animal, and the animal is then able to respond to stress. In addition, the expression of certain genes related to the immune profile was variable depending on the developmental stage of the larvae. The last hypothesis relies on the experimental protocol itself. Our experimental infection was performed in flasks without

renewing the seawater and without adding food contrasting with the study conducted by Burge and Friedman (2012) where those were renewed or fed daily. These conditions could have favored the disease, given that OsHV-1 mortality decreased with water renewal in *C. gigas* spat (Petton et al., 2015) and that energy reserves were diminished in infected oysters (Tamayo et al., 2014). To conclude with trial 2b, the significant decrease in the amount of OsHV-1 from 10^{+7} at day 5 to 10^{+4} at day 7 may be the consequence of the decreased replication of OsHV-1 because of the lack of live larvae between days 5 and 7 for both stocks (Figure 4C). This is in agreement with the findings obtained by Burge and Friedman (2012).

For the first time, 16-day-old larvae were evaluated for OsHV-1 resistance during 2c. These larvae exhibited moderate mortality, reaching 50% at day 7 for selected or unselected larvae under unchallenged conditions as well as for selected larvae under the challenged condition (Figure 3C). According to the quantification of OsHV-1, those findings were not related to OsHV-1. To explain this mortality, we hypothesized that in addition to the absence of feeding, the larvae were at a sensitive step of their lives, given that they were eyed larvae, and near metamorphosis at that. Nevertheless, when exposed to OsHV-1, unselected larvae exhibited higher mortality than selected larvae, which was related to OsHV-1 according to the OsHV-1 DNA detected at days 3, 5, and 7. At this stage, unselected larvae had a significant larger amount of OsHV-1 DNA than selected larvae (Figure 4C), strongly supporting the idea that genetic resistance was present at this stage of development.

In following up with our findings, the next step is to study the molecular mechanisms and protein expression in unselected and selected larvae before, during and after an experimental

infection with the OsHV-1 virus. It also appears that the larval model would be a powerful approach to understanding genetic resistance to OsHV-1 better as well as facilitating a selective breeding program. Indeed, the family that showed higher resistance to OsHV-1 infection could be identified a couple days after spawning, allowing it to reduce the span of the genetic evaluation, and thus decreasing the cost of the breeding program. Finally, monitoring oyster larvae through settlement to juveniles when exposed to OsHV-1 should also be useful to better understand the abundance of spatfall, which is of great importance for oyster farmers.

AUTHOR CONTRIBUTIONS

LD and JP designed the experiment. LD and ST produced the larvae. ST performed the infection experiments. ST, JP, and BM conducted the disease analyses. LD analyzed the data. LD, JP, and BM wrote and edited the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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