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Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian OsHV-1 µVar strain

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Abstract

In Australia the spread of the Ostreid herpesvirus OsHV-1 microvariant (OsHV-1 µVar) threatens the Pacific oyster industry. There was an urgent need to develop an experimental infection model in order to study the pathogenesis of the virus in controlled laboratory conditions. The present study constitutes the first attempt to use archived frozen oysters as a source of inoculum, based on the Australian OsHV-1 µVar strain. Experiments were conducted to test (i) the virus infectivity, (ii) the dose-response relationship for OsHV-1, and (iii) the best storage conditions to keep infective viral inoculum.

Intramuscular injection of a viral inoculum consistently led to an onset of mortality 48h post injection and a final cumulative mortality exceeding 90%, in association with high viral loads (1 x 10^5 – 3 x 10^7 copies of virus mg^{-1}) in dead individuals. For the first time an infective inoculum was produced from frozen oysters (tissues stored at -80°C for 6 months). Storage of purified viral inoculum at +4°C for 3 months provided similar results to use of fresh inoculum whereas storage at -20°C, -80°C and room temperature was detrimental to infectivity. A dose-response relationship for OsHV-1 was identified but further research is recommended to determine the most appropriate viral concentration for development of infection models that would be used for different purposes. Overall, this work highlights the best practices and potential issues that may occur in the development of a reproducible and transferable infection model for studying the pathogenicity of the Australian OsHV-1 strain in *C. gigas* under experimental conditions.

Keywords: Ostreid herpesvirus 1; *Crassostrea gigas*; experimental infection model; dose-response; inoculum preparation; storage conditions

Introduction

Episodic herpes-like virus infections in Pacific oysters *Crassostrea gigas* have been reported in many European countries (France, United Kingdom, Jersey, Ireland, Spain and The Netherlands) and also in the United States of America (Renault 1994, Renault & Novoa 2004, Davison et al. 2005, Friedman 2005, Schikorski, Renault, et al. 2011). Since 2008 mortality outbreaks have mainly been associated with the presence of a new herpesvirus strain (OsHV-1 µVar) infecting Pacific oysters during the summer period, concomitantly with a rapid increase of seawater temperature (Renault 2011).

In late 2010, a few months after having decimated farms in bays on the North island of New Zealand, OsHV-1 µVar occurred for the first time in Australia in a small population of commercially farmed triploid Pacific oyster spat in Woolooware Bay within the Georges River/Botany Bay estuary, New South Wales (NSW) (Jenkins et al. 2013). In the present paper the term "OsHV-1" refers to OsHV-1 µVar. Epidemiological investigations were conducted in Botany Bay in order to identify critical risk factors leading to mortality (Paul-Pont et al. 2013a, b). A little over two years later the first sign of spread of the virus was identified in the Hawkesbury River estuary (located 50 km north of Botany Bay) with OsHV-1 µVar related mass mortalities observed in commercial *C. gigas* leases (Paul-Pont et al. 2014). Due to the threat that OsHV-1 µVar poses to the entire Australian Pacific oyster industry, research activities were first directed towards (i) a mollusc breeding program to develop disease resistant Pacific oysters and (ii) the conduct of large-scale epidemiological investigations to identify the main risk factors for the disease outbreaks. In parallel, the development of a reproducible and transferable experimental infection model was crucial to study the virus and the mechanisms underlying infection and death in detail (pathogenicity, host immunity, risk factors). Indeed, important epidemiological features differ between the European and the Australian OsHV-1 µVar related mortality outbreaks (threshold temperature, window of infection, spatial and temporal distribution of mortality) (Pernet et al. 2012, Paul-Pont et al. 2013a, b, 2014), which underline the need to study the Australian OsHV-1 µVar strain in local controlled laboratory conditions.

The present study describes the first experiments to set up a reproducible laboratory based experimental infection using the Australian OsHV-1 µVar strain purified from naturally infected oysters. Successful infection models using intramuscular injection that had already been developed in France (Schikorski, Faury, et al. 2011, Schikorski, Renault, et al. 2011) were used as a basis to develop a "local" infection model. Four experimental infection trials were performed in order to test (i) the infectivity of the Australian OsHV-1 µVar strain using infected material sampled from the Georges River during the summer of 2011/2012 (experiments 1a and 1b), the dose-response relationship for OsHV-1 (experiment 2) and the best storage conditions to keep purified viral inoculums (experiment 3). Daily mortality was recorded for a period of 10 days. Viral concentrations in oyster tissues and seawater were also measured by real-time quantitative PCR during the time course of the experiments.

Material and methods

1. Experiments

Four experiments were sequentially performed:

- Experiments 1a and 1b: The aim was to assess whether the disease was reproducible in laboratory conditions using the Australian OsHV-1 µVar strain (temporally replicated experiments);

- Experiment 2: The aim was to determine a dose-response relationship between the quantity of viral particles injected and the cumulative mortality of oysters;

- Experiment 3: The aim was to identify the appropriate storage conditions required to maintain the viability of the Australian OsHV-1 µVar strain as a purified inoculum under laboratory conditions.

2. Origin of oysters and acclimation period

All naive oysters used in this study were hatchery single seed triploid oysters (Shellfish Culture, Tasmania) and came from the same batch (11 month old; size 40-50 mm length). Naive oysters were harvested from Porto Bay in the Hawkesbury River (Oyster Leases 68/178 and 85/023), which had been free of OsHV-1 until 2013. Indeed, as part of another research project, a monitoring program was set up in the Hawkesbury River from September 2011 to September 2012 and confirmed the absence of OsHV-1 during this period (Paul-Pont et al., 2014). Additionally, n=30 oysters were sampled and tested negative for OsHV-1 using the TaqMan assay (Paul-Pont et al. 2013b) prior to each experiment.

The oysters were harvested at different times depending on the experiment: July 2012 (Experiment 1a), August 2012 (Experiments 1b and 2) and September 2012 (Experiment 3). Although no significant difference in size was observed in the oysters sampled from July to September (data not shown), it is reasonable to assume that oysters were not exactly identical among experiments as they aged over time. However as the sampling time of oysters was nested within experiment, there was no bias introduced in relation to the time of sampling.

The oysters were transferred to the acclimation tanks within less than 12h after the sampling and the water temperature was set up to correspond to the environmental conditions in the field (15-18°C) while the salinity was fixed at 30 ppt. The oysters were acclimatised for one week in 25 L tanks set up on a recirculation system connected to chillers, biofiltration and UV light. The water temperature in the tanks was increased by 1°C per day until it reached 22°C used during the experiments. Oysters were fed daily by addition of an adequate quantity (4×10^5 - 1.6×10^7 cells/ml) of shellfish diet 1800 (Instant Algae®, Reed Mariculture, USA) containing a mix of *Isochrysis sp.*, *Pavlova sp.*, *Thalassiosira weissflogii* and *Tetraselmis sp*. No mortality was recorded prior to the experiments.

3. Inoculum preparation

Oyster tissue homogenate from OsHV-1 µVar infected and non-infected Pacific oysters were used to prepare infected and control inoculums, respectively. OsHV-1 infected Pacific oysters (12 month old; length: 79.5 ± 4.6 mm, mean ± SD) were collected from the Georges River, NSW, Australia during a disease outbreak on the 24th November 2011. Control oysters (6 month old; length: 52.7 ± 8.8 mm, mean ± SD) were collected from the Hawkesbury River on the 19th December 2011. Both batches of oysters (*i.e.* infected and control) were previously tested by PCR to confirm their respective status (*i.e.* infected and control) and they were stored at -80 °C prior to being used for each experiment. For experiments 1 and 2 five oysters from each batch were removed from storage at -80°C one day prior to the commencement of the experiment and were thawed at 4°C overnight. The following day, each batch of 5 oysters was processed separately with sterile materials, and working benches were carefully disinfected with 1 % Virkon® between batches to prevent cross contamination. Oysters were opened by removing the superior valve, and a small piece of mantle (0.08-0.12 g) was sampled from each oyster and placed at -80°C before DNA extraction and OsHV-1 detection by real-time PCR. The rest of the gills and mantle were dissected and pooled together (total weight: 10-12g) into a 50 mL sterile tube maintained in wet ice. These tissues were weighed and diluted by adding 10 volumes of 0.22 µm filtered synthetic seawater (FSSW) to the tube. Tissues were homogenised using a MiniMix® stomaching machine (Crown Scientific, Australia) stomaching machine for one minute at maximum speed. After centrifugation (1000g, 5min, 4°C) supernatant was placed in a new sterile tube and diluted with 3 volumes of FSSW. Finally, the clarified tissue homogenate was filtered consecutively under aseptic conditions in a Class 2 Biosafety cabinet using syringe filters at 8 µm, 0.45 µm and 2 x 0.22 µm pore sizes. The purified homogenate, or inoculum, was maintained on ice prior to the injection.

For experiment 1 the inoculum was freshly prepared. Syringes containing 100 µL of each purified homogenate (control and infected) were prepared in the Class 2 Biosafety cabinet and maintained

on ice prior to the injection. The concentrations of the infected inoculums were 1.4 x 10^6 and 1.3 x 10^7 viral DNA copies per ;mL in experiments 1a and 1 b respectively. No viral DNA was detected in the control inoculums.

For experiment 2 the inoculum was freshly prepared and five concentrations were tested (pure at 5 x 10^6 viral DNA copies per mL, and 10-fold serial dilutions: 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in parallel with a control group. Accordingly, the pure inoculum was diluted 4 times with FSSW before being filtered twice using 0.22 µm pore sizes syringe filters prior to injection. Syringes containing 100 µL of each purified homogenate were prepared in the Class 2 Biosafety cabinet and maintained on ice prior to the injection.

For experiment 3, the inoculum that had been freshly prepared in July 2012 (experiment 1) and dispensed into four tubes (5 mL) separately to be kept at room temperature (RT), +4°C, -20°C, -80°C and liquid nitrogen was used. In early October 2012 (+ 3 months) the different inoculums were thawed overnight at + 4°C (if required) and filtered twice using 0.22 µm pore sizes syringe filters prior to injection. Syringes containing 100 µL of each purified homogenate were prepared in the Class 2 Biosafety cabinet and maintained on ice prior to the injection. The viral DNA concentrations of each inoculum are listed in Table 2.

4. Experimental infection

Each intramuscular (IM) injection was carried out on the same day as the homogenate preparation, except for experiment 3 where stored inoculums were used.

The IM injection was carried out using 8-10 oysters per treatment per 25L tank and was conducted in triplicate (n=3 replicate tanks containing a total n=30 oysters per treatment for experiments 1 and 3, and n=24 oysters per treatment for experiment 2). Oysters were placed out of the water (air temperature 22°C) for 24h prior to immersion in a magnesium chloride solution ($MgCl_2$; 50 $g.L^{-1}$) in seawater (1v)/distilled water (4v) for between 1 and 4 hours. Once relaxed, 100 µL of filtered tissue homogenate was injected into the adductor muscle of each oyster. The control and the infected oysters were injected on separated benches to prevent cross contamination and the inoculated oysters were put directly back in the water without any food supply or filtration system. This design ensured that (1) no water was shared among tanks due to recirculation; (2) the water temperature was uniform between tanks as it was controlled by equilibration with the air temperature (21.7 ± 1.2 °C, mean ± SD). The salinity was monitored daily and stayed stable (30 ppt). Water quality parameters (ammonia, nitrate, nitrite, pH) were monitored daily. In experiment 1b the water was occasionally changed to maintain water quality in acceptable ranges.

All experiments were conducted in a dedicated PC2 facility at the University of Sydney in accordance with the PC2 biosecurity regulations and protocols.

5. Sampling strategy

A water sample was collected daily from each tank for PCR analysis (volume sampled=4mL, volume tested by PCR=200µL). Mortality was monitored daily for 10 days and no active sampling was performed on live oysters. Mean daily mortality ± SD was calculated among the three replicate tanks. Dead oysters were systematically removed from the tanks during the time course of the experiment and a piece of gill and mantle (0.08-0.12 g) was sampled from each dead individual. Control and surviving oysters were all sampled and sacrificed at the end of experiment. All tissue and water samples were frozen at -80°C prior to DNA extraction and OsHV-1 quantification by real-time PCR.

6. Detection of OsHV-1 by real-time PCR in water and oyster tissues

    a. Tissue homogenisation

A piece of gills and mantle (0.08-0.12 g) was dissected from individual oysters using disposable sterile instruments that were changed between each oyster. After addition of 9 volumes of sterile distilled water, tissues were ground by bead-beating (Speed 6.5 m/sec; Time: 15 sec) (Fastprep System, MP Biosciences, USA). Tissue homogenates were then clarified by centrifugation at 900 × *g* for 10 min in a microcentrifuge. Clarified tissue homogenates were stored at − 80 °C until required.

b. DNA extraction

Viral DNA extraction was performed via magnetic beads using a MagMax-96 Viral RNA Isolation Kit (Ambion, USA) following the manufacturer's recommended protocol, in a MagMax Express-96 magnetic particle processor with disposable tip combs and standard 96-well processing plates (Appiled Biosystems, USA). Extraction was performed on a 50 µL aliquot of clarified tissue homogenate and a 200 µL aliquot for water samples.

c. Real-Time Polymerase Chain Reaction (PCR)

The detection of OsHV-1 µVar DNA by real-time PCR was adapted from a previously published protocol (Martenot et al. 2010) and fully detailed in (Paul-Pont et al. 2013b). The detection limit was evaluated at 3 copies per mg of tissue and the quantification limit at 12 copies per mg of tissue based on guidelines previously published (Bustin et al. 2009, Martenot et al. 2010). A valid run was defined as a run exhibiting no amplification of the negative control, amplification of the positive control (Ct within the range of the standard curve) and a standard curve with $r^2 > 0.95$ and efficiency between 90 and 110%. Threshold setting for each run was manually locked in based on the standard curve series. A sample was defined as positive when both replicates exhibited an exponential accumulation of fluorescence and a valid cycle threshold.

7. Statistical analyses

Summary statistics and box-and-whisker plots of time to mortality by treatment were initially created to make a preliminary assessment of the effect of infection on mortality. Kaplan Meier curves were created to compare survival between treatment groups for each experiment. Marginal cox models were then fitted to estimate hazard ratios after adjusting for the tank intra-cluster correlation. This approach uses a robust sandwich covariance matrix estimate to adjust for clustering (Lin, 1994). The proportional hazard assumption was evaluated graphically by creating log cumulative hazard plots and by testing time dependent covariates with log of time. Hazard ratios and their 95% confidence intervals were presented. All the analyses were conducted using SAS statistical program (© 2002-2010 by SAS Institute Inc., Cary, NC, USA) but the Kaplan-Meier curves were created using ggsurv function in R statistical package version 3.0.1 (© The R foundation for statistical computing).

For the calculation of the mean viral copies/mg ± SD of tissues the authors firstly confirmed the absence of any significant tank effect (using an ANOVA test, $p > 0.05$; STATISTICA 10 software for Windows) before considering all replicate oysters, regardless of the tank number.

Results

1. Mortality

During experiment 1a (July), oysters infected by intramuscular injection of OsHV-1 suspension were characterized by a sudden mortality reaching 70% at 48 hours post-injection (pi). At 72h pi, the cumulative mortality was 90% and reached 97% at 96h pi. Only one infected oyster survived and was eventually sacrificed at the end of the experiment (240 hours pi) for OsHV-1 quantification. The mortality of control oysters was nil, so all (n=30) were sacrificed and tested by real-time PCR at the end of the experiment. A similar pattern of mortality was observed in experiment 1b (August), with an onset of mortality 48h pi and a cumulative mortality of 96% was reached at 96h pi (Figure 1). All

OsHV-1 injected oysters were dead at 144h pi while no mortality was observed in the control group. The only difference in the mortality pattern between the two experiments concerned the cumulative mortality observed at 48h pi: 70% in experiment 1a and only 19% in experiment 1b, *i.e.* it was a little slower to commence in experiment 1b. Survival analysis indicated significant differences in survival probabilities between the two groups in both trials ($p < 0.001$) but hazard ratio confidence intervals could not be calculated due to nil mortality in control groups in both trials (Figure 1).

In experiment 2 there was a clear dose-response relationship between the quantity of viral particles injected into the adductor muscle and the survival of oysters (Figure 1). Mortality was almost nil in the control group (one oyster died at 72h pi) and was therefore deleted for calculating hazard ratios. Mortality remained low in the highest dilution group (10^{-4}) (two oysters died at 144 and 168h pi), while heavy mortalities reaching up to 88-96% were observed in the groups injected with pure inoculum or the lowest dilutions (10^{-1} and 10^{-2}) (Figure 1). Dose response relationship was also evident from the hazard ratios presented in Table 1, which indicate that compared to the reference group injected with 5×10^2 copies per mL of virus, the other groups injected with greater quantities of virus had a greater hazard of death and the hazard was maximum in the group injected with 5×10^6 copies of virus per mL.

The survival analysis results for the experiment 3 are presented in Figure 1 and Table 1. The injection of the inoculum kept at +4°C for 3 months induced a significant mortality, similar to the mortality observed after injection of the fresh inoculum during the experiment . There was a cumulative mortality of 94% after 96h and 100% by the end of the experiment (240h pi). Survival in the +4°C group was significantly lower than all other groups with hazard ratios of 10.31 (95% confidence interval [CI]: 6.0, 17.8) compared to the room temperature group; 28.15 (95% CI: 18.5, 42.8) compared to the -20°C group; and 27.33 (95% CI: 12.4, 265.0) compared to the -80°C group (Figure 1, Table 1). The hazard of death in the group injected with the inoculum kept at room temperature was significantly higher than the groups injected with inoculums kept at -80°C group (hazard ratio: 5.56; 95% CI: 1.3, 24.1) and -20°C group (2.7; 95% CI: 2.0, 3.8). However, survival was not significantly different between -20°C and -80°C groups (Figure 1). These last two inoculums did not induce any substantial mortality as demonstrated by the cumulative mortalities of 3% and 7% at 10d pi, respectively. The injection of the inoculum kept in liquid nitrogen did not induce any mortality. No mortality was observed in the control group (Figure 1). Both of these groups were excluded for calculation of hazard ratios.

2. Viral concentrations in oyster tissues and in seawater

OsHV-1 was never detected in control oysters, confirming the absence of cross-contamination during the experiments.

For experiment 1a the viral loads in dead oysters sampled during the first 96h post-injection, during which time 29 out of 30 oysters died, were between 3.7×10^5 and 2.5×10^7 copies mg^{-1} of tissue (Figure 2). As each oyster from the OsHV-1 group was injected with 100 µL of an inoculum of 1.4×10^6 copies mL^{-1}, the initial dose administered to these individuals was 1.4×10^5 copies. Viral loads measured in the dead individuals were much higher than the initial dose injected and thus confirm an efficient replication of viral particles in oyster tissues (Figure 2). The only infected survivor was sampled at the end of the experiment (240h pi) and presented a lower viral load in its tissue (1.1×10^3 copies mg^{-1}). Viral loads in infected tissues were significantly lower at 240h pi compared to all other time points ($P<0.001$).

The viral concentrations in water increased from 9.0×10^4 to 1.8×10^6 copies mL^{-1} at 24 and 48h pi, respectively, before progressively decreasing to 2.5×10^2 copies mL^{-1} (Figure 2). For experiment 1b another PCR test (Pépin et al., 2008) was employed, therefore the data cannot be directly compared to the other experiments and are not presented in this paper. Even though occasional water changes at day 3 and 4 pi modified the viral loads in water, this did not affect the overall kinetics of infection recorded in oysters (data not shown).

For experiment 2, different concentrations of inoculum were administered ranging from 5.1 x 10^6 (pure inoculum) to 5.1 x 10^2 (most diluted inoculum – 10^{-4}) copies mL^{-1} in parallel to a control group injected with a virus-free inoculum. No virus was detected in the control group or in the oysters injected with the most diluted inoculum (inoculum 10^{-4}). In all the other conditions (P, 10^{-1}, 10^{-2}, 10^{-3}) the viral loads in dead oysters sampled during the experiment (i.e. all oysters sampled before 240h pi) were between 1.1 x 10^5 and 1.2 x 10^7 copies mg^{-1} regardless of the concentration of the inoculum used for the injections, except for one individual injected with inoculum 10^{-2} (5.1 x 10^4 copies mL^{-1}) sampled at 168h pi (viral load in tissue: 2.5 x 10^4 copies mg^{-1}) (Figure 3). Similarly to experiment 1a, the viral loads measured in the surviving oysters actively sampled at 240h pi were much lower than the concentration measured in the dead individuals and in the corresponding inoculum (except for the surviving oysters injected with the inoculum 10^{-3}) (Figure 3).

The viral concentrations in seawater were relatively stable during the first 120h pi and were between 5.1 x 10^3 and 9.6 x 10^4 copies mL^{-1}, regardless of the concentration of the initial inoculum. A decrease in the viral concentration in seawater was observed towards the end of the experiment (> 120h pi) as the tanks were progressively emptied by removing the dead individuals.

For experiment 3, the viral concentrations measured in the stored inoculums were systematically lower than the concentration measured in the fresh inoculum in July 2012 (1.3 x 10^6 copies mL^{-1}), the lowest concentrations being found in the inoculums kept at room temperature and 4°C and the highest concentrations being observed in the frozen inoculums (-20 and -80°C) (Table 2). No viral DNA was detected in the dead (sampled during the time course of the experiment) and live (sampled at 240h pi) oysters injected with the viral inoculums kept at -20°C, -80°C and in liquid nitrogen. Among the five dead oysters that died up to 72h after having been injected with the inoculum kept at RT, three of them were negative for the viral DNA and the two remaining oysters demonstrated 1.2 x 10^1 and 2.4 x 10^2 copies mg^{-1} (both were sampled at 48h pi). The only group that demonstrated significant mortalities corresponded to the oysters injected with the inoculum kept at +4°C (Figure 2) and the viral loads measured in the dead individuals varied between 6.7 x 10^5 and 3.0 x 10^7 copies mg^{-1} (Figure 4). The virus was only detected in the seawater of the tanks corresponding to the +4°C storage conditions and the concentrations were relatively stable over time, ranging from 2.2 x 10^3 to 7.7 x 10^4 copies mg^{-1} (Figure 4).

Discussion

This work constitutes the first attempt to achieve a reproducible laboratory based experimental infection using the Australian OsHV-1 µVar strain purified from naturally infected oysters. It is the first report to confirm that OsHV-1 µVar remains viable in oyster tissues stored at -80°C, and such oysters can be used as a source of virus for infection trials.

1. Mortality kinetics

For all experiments intramuscular injections of pure viral inoculums prepared from Pacific oysters naturally infected by the Australian OsHV-1 µVar strain led to high cumulative mortalities (> 90% at the end of each experiment) associated with high viral loads in oysters. These results confirm active replication of the virus in experimentally infected animals as previously reported in other studies using the French OsHV-1 µVar strain (Schikorski, Faury, et al. 2011, Schikorski, Renault, et al. 2011, Segarra et al. 2014). Even though intramuscular injection does not reflect a "natural" infection as it overcomes the natural barriers (mucus, epithelia), this method was preferred over a cohabitation assay in order to obtain a standardized protocol, *i.e.* having all oysters infected at the same time with a similar dose of viral particles. The overall kinetics of mortality were similar among all individuals that had received a pure and fresh viral inoculum of similar concentration (1.3 – 5.1 x 10^6 copies mL^{-1}; Figures 1, 2, 3 and 4) with an onset of mortality at 48h pi and a cumulative mortality exceeding 90% after 7 days of exposure. This result is also in accordance with previous studies using

the intramuscular injection method and conducted on 7-12 month old Pacific oysters (Schikorski, Faury, et al. 2011, Segarra et al. 2014). No mortality was observed at 24h pi, which might be due to the time needed for viral replication in oyster tissues leading to irreversible cell damage and oyster mortality as suggested by Sauvage et al. (2009). Segarra et al. (2014) detected the presence of OsHV-1 mRNAs in spat tissues as soon as 2, 4 and 18h post injection suggesting that the virus starts replicating in the early hours of the infection but the first mortalities were only observed at 42h pi.

2. Dose-response effects

A dose-response relationship between the concentration of viral particles in the inoculum and the percentage of mortality at 10d pi was identified through the results of the third experiment. Indeed, at 10d pi high cumulative mortalities (> 85%) were observed in oysters injected with concentrated inoculums ($5.1 \times 10^4 – 5.1 \times 10^6$ copies mL^{-1}), while intermediate (63%) and insignificant (<10%) cumulative mortalities were recorded in oysters injected with less concentrated inoculum (5.1×10^3 and 5.1×10^2 copies mL^{-1}, respectively) (Figure 1). This result suggests that a sufficient initial dose of viral particles is needed to trigger high mortalities in Pacific oysters. Normand et al. (2014) showed great differences in mortality between two groups injected with a virulent and a non-virulent inoculum at 2×10^9 and 4×10^6 copies of viral genomes μL^{-1} and prepared from dying and apparently healthy oysters, respectively. However, it is unclear whether the differences in mortality were only due to the virulence of the virus or whether the concentration of the inoculum might also have played a role. In our experiment, the same inoculum was diluted serially 10 fold several times with FSSW and injected at the same time in all groups. Therefore it is clear that the differences in cumulative mortality were due only to the concentration of the inoculum and not to a potential difference in virulence. This result allows the identification of an appropriate inoculum concentration ($1\text{-}5 \times 10^3$ copies mL^{-1}) leading to slow mortality kinetics and intermediate cumulative mortality, which might be necessary in order to conduct research on the fine metabolic pathways in response to infection in live hosts prior to mortality, or to measure genetic effects. The study of viral or host transcripts is likely be modified in moribund/dead individuals due to degradation processes (Segarra et al. 2014).

3. Mortality pattern: comparison with other studies

Discrepancies occur in the literature when comparing the initial inoculum concentration and the mortalities obtained at 10d pi after intramuscular injection: Segarra et al. (2014) observed a cumulative mortality of 63% at 90h pi after the injection of 1.2×10^5 copies into 7 month old oysters; Normand et al. (2014) did not observe high mortalities (maximum cumulative mortality of 15% after 7 days of challenge) after the injection of 2×10^9 copies into 8 month old oysters; and Schikorski et al. (2011) observed an extreme mortality (>90%) after the injection of 1.5×10^7 copies into 12 month old oysters, which is in accordance with the results of the first two experiments presented in this paper. This variability in the short term mortality kinetics after intramuscular injection of a concentrated OsHV-1 inoculum highlights the potential issues and difficulties that may occur in the development of a reproducible and transferable infection model. Mainly, these issues concern (i) the variation in the natural host due to the utilization of different oyster batches for each experiment; (ii) the experimental parameters such as water renewal, density of infected host and nutrition; and (iii) the variation in the viral inoculum itself (strain, virulence). Natural variation in the resistance to pathogens, and particularly OsHV-1, is commonly observed in Pacific oyster populations and is mainly related to age, physiology, individual genetic basis and life history (Pernet et al. 2012, Dégremont 2013, Dégremont et al. 2013, Normand et al. 2014, Green et al. 2014). Additionally, the density of the infected population within a tank may also greatly affect the kinetics of the disease. In the present study a density of 8-10 oysters for 25 L of seawater (without food supply) was used whereas Normand et al. (2014) placed 40 infected oysters in 20L tanks in a flow-through system with food, and Schikorski et al. (2011) used 100 naive oysters in cohabitation with 40 moribund animals in 25 L tanks. The water renewal regime was also different among studies. Therefore some

discrepancies between studies and/or infection trials seem quite inherent, and some precautions must be taken when comparing data from different studies. These discrepancies can also be explained by the variation in the viral inoculum itself (strain, virulence) as demonstrated by Normand et al. (2014) as well as by its conservation and stability over time, as demonstrated by the results of the third experiment.

4. Storage conditions

The storage of the viral inoculum at +4°C for a period of 3 months allowed the viral particles to remain highly infectious, as suggested by the high cumulative mortality obtained in this treatment, which was similar to the cumulative mortality obtained in July with the fresh inoculum (Figure 1). On contrary Corbeil et al., (2012a) demonstrated a loss of infectivity over time when the Abalone herpesvirus (AbHV) inoculum was held at +4°C. In addition, the authors mentioned that keeping AbHV inoculum in liquid itrogen prolongs viral infectivity for longer time periods (at least 21 months) than storage at -20°C and -80°C (Corbeil et al., 2012b). This is different from our results as the injection of OsHV-1 viral inoculums kept at room temperature, -20°C, -80°C and liquid nitrogen for 3 months did not lead to any significant mortality (< 14%), regardless of the concentration of viral particles in each inoculum (Figure 1; Tables 1 and 2). However, as no cryoprotectant was employed here, this experiment should be repeated with additional treatments (*i.e.* test of a range of cryprotectants and protocols for progressive reduction of temperature). The experiment was purposely conducted without the use of cryoprotectant to compare with the success of the previous infection trials (experiments 1a and 1b) for which frozen oysters (temperature: -80°C; duration: 6 months) were employed to prepare the viral inoculums. To our knowledge it is the first time that frozen tissues have been used as a source of infective viral particles; all previous studies with OsHV-1 used fresh oysters collected from infected areas in the field or experimentally infected (Renault et al. 2011, Schikorski et al. 2011, Schikorski, Renault, et al. 2011, Burge & Friedman 2012, Normand et al. 2014, Green et al. 2014). The use of archived frozen oysters as source of infective particles constitutes a great step forward, allowing the conduct of experimental infections all year long even when the disease is not active in the field and fresh infected oysters are not available. This result also raises a question about the consequences of importing frozen oysters from infected areas/states/countries to non-infected locations: is this a possible means for spread of virus if infected frozen products may end up in coastal waters, for example through oysters being diverted from the human food chain to be used as bait, or shells being discarded in coastal waters from pleasure boats? Therefore studies are required to assess the viability of OsHV-1 in commercial fresh-frozen oysters.

5. OsHV-1 load in oysters tissues

The viral concentrations measured in the gills and mantle of dead oysters collected during each infection trials were high and ranged between 1.1×10^5 and 3.0×10^7 copies mg^{-1}, regardless of the infection trial, the time of collection and the initial concentration of viral particles in the inoculum, with a few exceptions (2 dead oysters from treatment 10^{-4} in experiment 2, and 1, 2 and 5 dead oysters from treatments “-80°C”, “-20°C” and “RT” in experiment 3, respectively). This result suggests that regardless of the time post infection and the quantity of viral particles injected into the animal, the quantity of viral particles must reach a threshold in oyster tissues before inducing death. This is consistent with previous field and lab studies demonstrating that viral concentrations exceeding 10^6 - 10^7 copies mg^{-1} are systematically associated with moribund/dying/dead oysters (Pepin et al. 2008, Oden et al. 2011, Paul-Pont et al. 2014). All oysters that survived the injections and were sacrificed alive at the end of the experiments (240h pi) demonstrated significantly lower viral concentrations in their tissues, below 3.1×10^3 copies mg^{-1}, with a few exceptions (2 x 2 live oysters from treatments “10^{-3}” and “10^{-1}” in experiment 2). The low viral loads found in the survivors is in accordance with Oden et al. (2011) who defined a viral load threshold of 8.8×10^3 copies mg^{-1} below which the risk of mortality due to the disease was null.

6. OsHV-1 variation in seawater

During the first 24h the average viral concentrations in water increased from 0 (data not shown) to 9.0×10^4 copies mL^{-1} in experiment 1a, 5.1×10^3, 1.6×10^4 and 1.7×10^4 copies mL^{-1} respectively in 10^{-2}, 10^{-1} and pure inoculum conditions in experiment 2, and 2.2×10^3 copies mL^{-1} in experiment 3 (+4°C condition) (Figures 4, 5 and 6). Regardless of the infection trial or the initial concentration of viral particles in the inoculum, the average viral concentrations measured in water remained relatively stable over the first 120h and ranged between 2.2×10^3 and 1.8×10^6 copies mL^{-1}. The decrease in viral concentrations in water observed towards the end of the experiments (>120h pi) were likely to be due to the progressive removal of the dead individuals. These results are in accordance with Schikorski et al. (2011) who demonstrated an increase in the viral concentrations in water up to 1×10^5 copies mL^{-1} during the 48h following the introduction of injected oysters to the tanks. A decrease in the viral concentration in water was also observed after the removal of the injected oysters.

Conclusion

In conclusion, the present work demonstrated that infection trials can be successfully developed in "local" conditions using the Australian OsHV-1µVar strain and healthy virus-free Pacific oysters from a non-infected estuary. However great precautions must be taken regarding (i) the source for the viral inoculum and its stability over time; (ii) the characteristics (age, physiology, genetics, life history) of the oysters used for the infection trials; and (iii) the experimental conditions (nutrition, infected host density, water renewal) in order to be able to develop a reproducible and transferable infection model. Conservation of the viral inoculum at +4°C provided the best outcome with similar cumulative mortality observed in comparison with the results of the first experiment performed 3 months before with the fresh inoculum. A clear dose-response relationship for OsHV-1 was identified and further research is recommended to determine the most appropriate viral concentration to use for the development of an infection model for different purposes. Finally, for the first time an infective inoculum was produced from frozen oysters (-80°C for at least 6 months), thereby enabling infection trials to be conducted at any time of year. However, we raise a question about the potential for persistence of live OsHV-1 in fresh-frozen commercial oysters, and whether this could be a mean of translocation of virus.

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Figures

Figure 1. Survival analyses to compare mortality of Pacific oysters *Crassostrea gigas* exposed to different treatments in experiments 1a, 1b, 2 and 3.

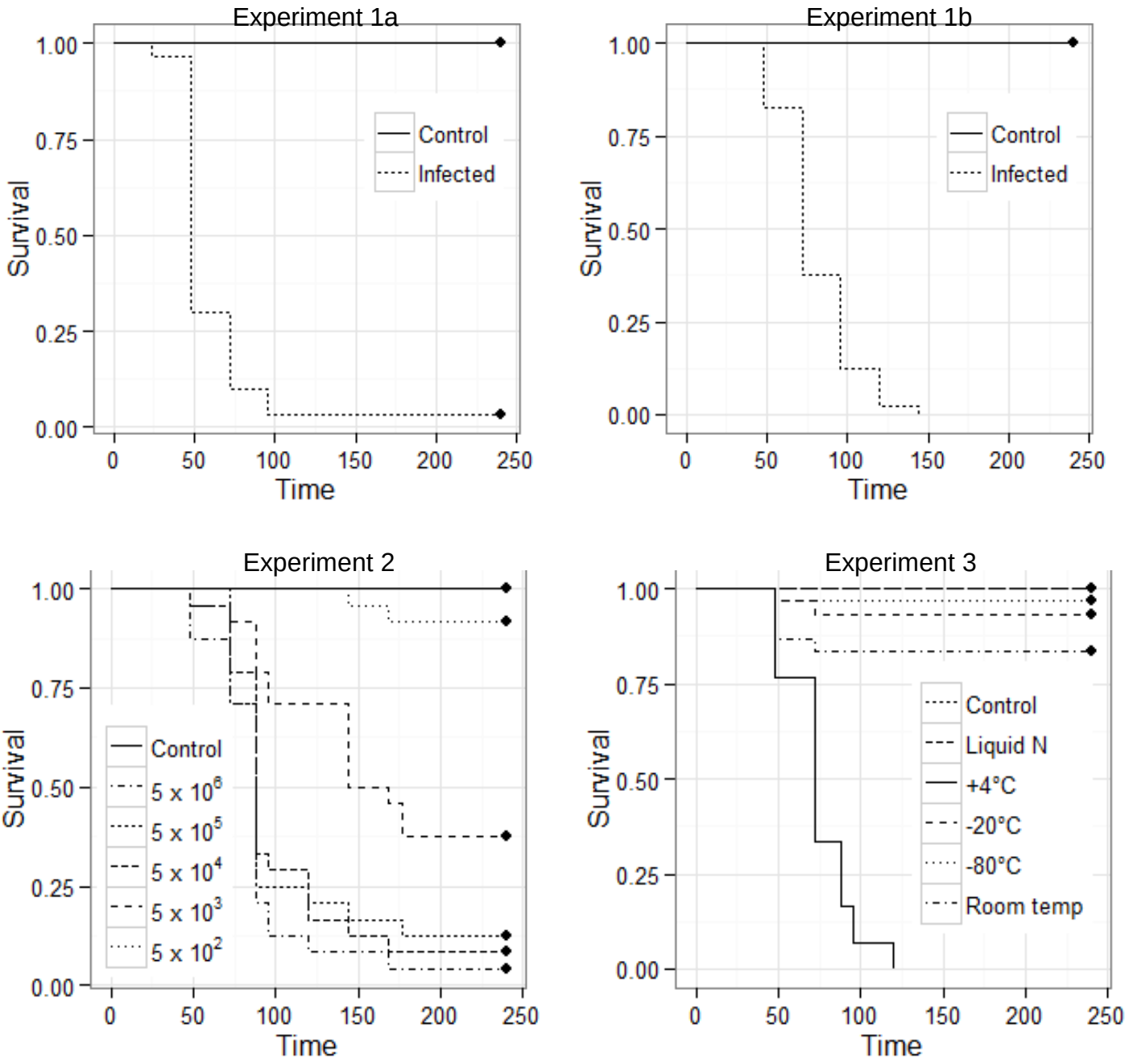

Figure 2. Viral loads in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) sampled in experiment 1a. All of the oysters sampledwere dead individuals that were systematically removed from the tanks, except for one live individual sampled at 240h post injection (black bar). Results are expressed as copies per mg for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number above each bar represents the number of oysters analyzed at each time.

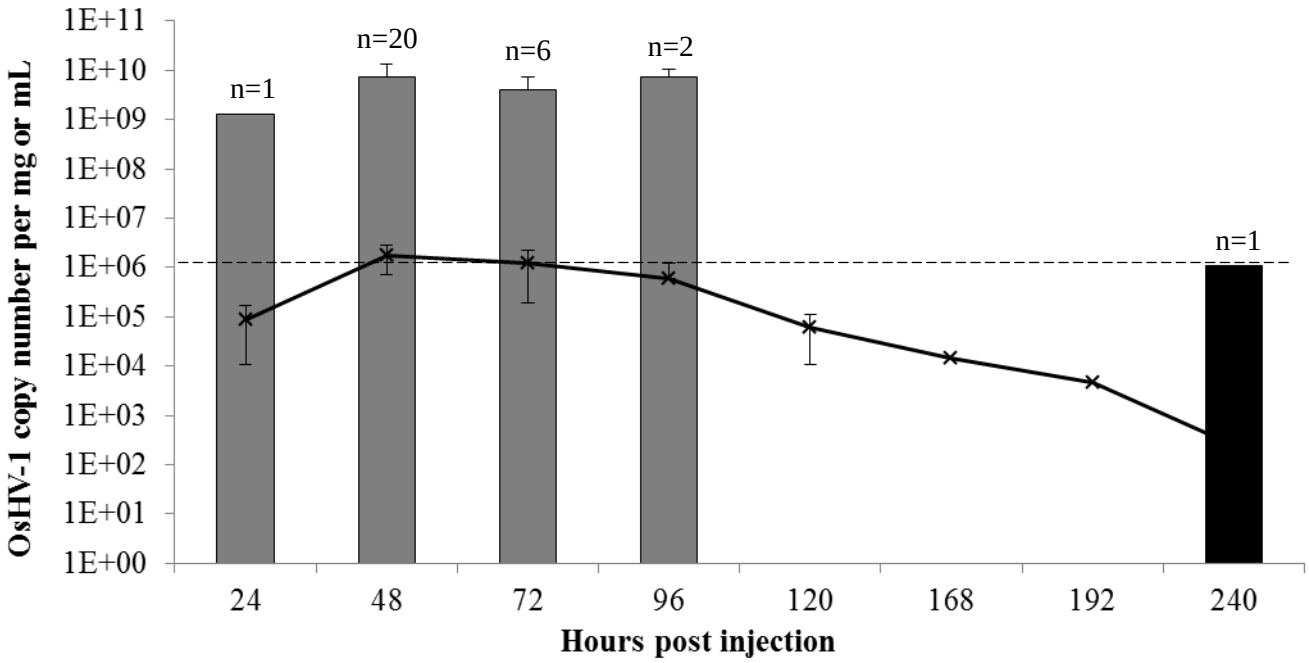

Figure 3. Viral load in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) sampled in experiment 2. Different concentrations of viral inoculum were used: pure inoculum at 5.1 x 10^6(a) and serial dilutions: 5.1 x 10^5 (b), 5.1 x 10^4(c) and 5.1 x 10^3(d) copies mL^{-1}. All of the oysters sampled were dead individuals that were systematically removed from the tanks, except for the individuals sampled at 240h post injection (black bars). Results are expressed as copies per mg for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number above each bar represents the number of oysters collected at each time.

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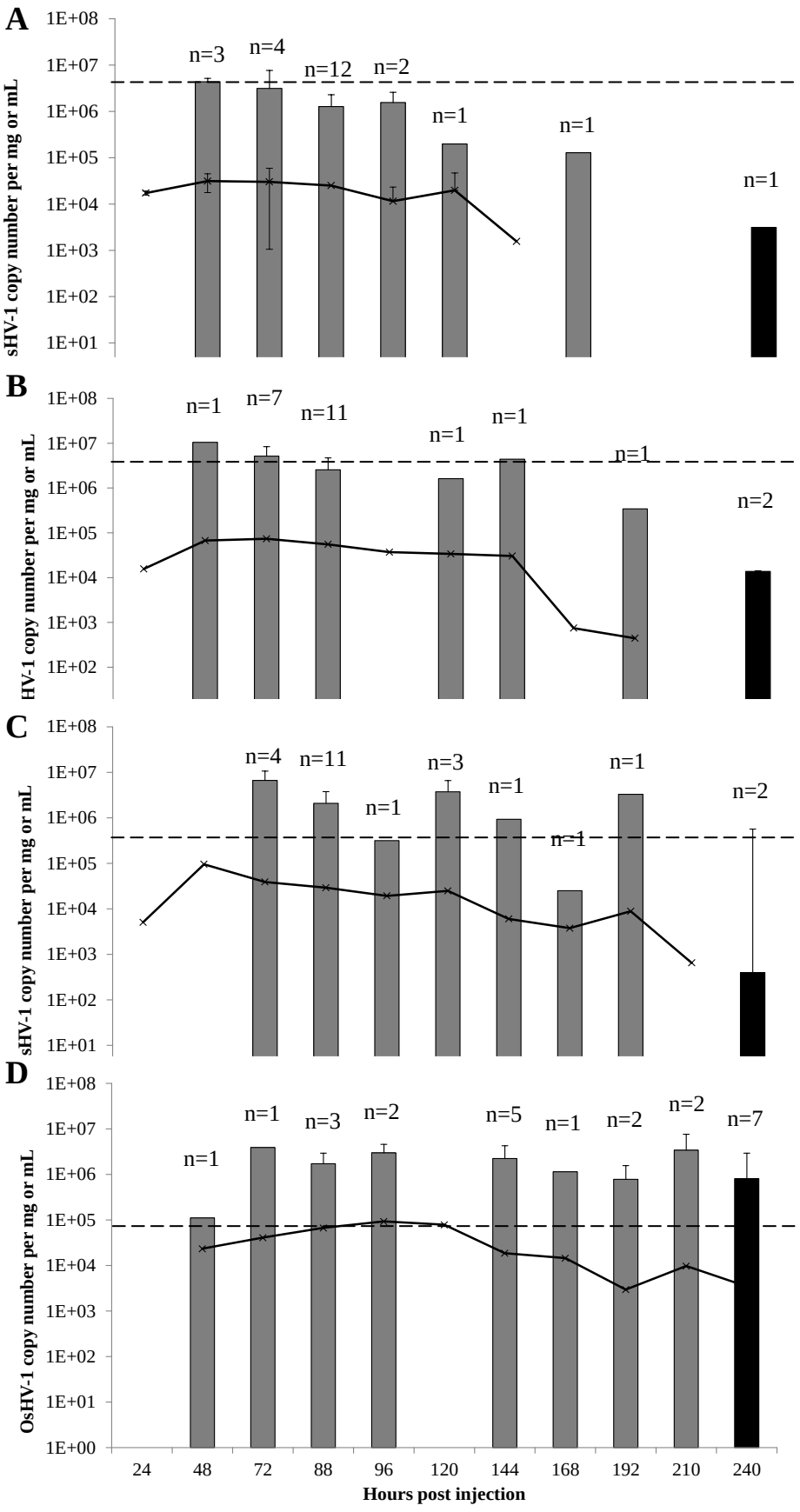
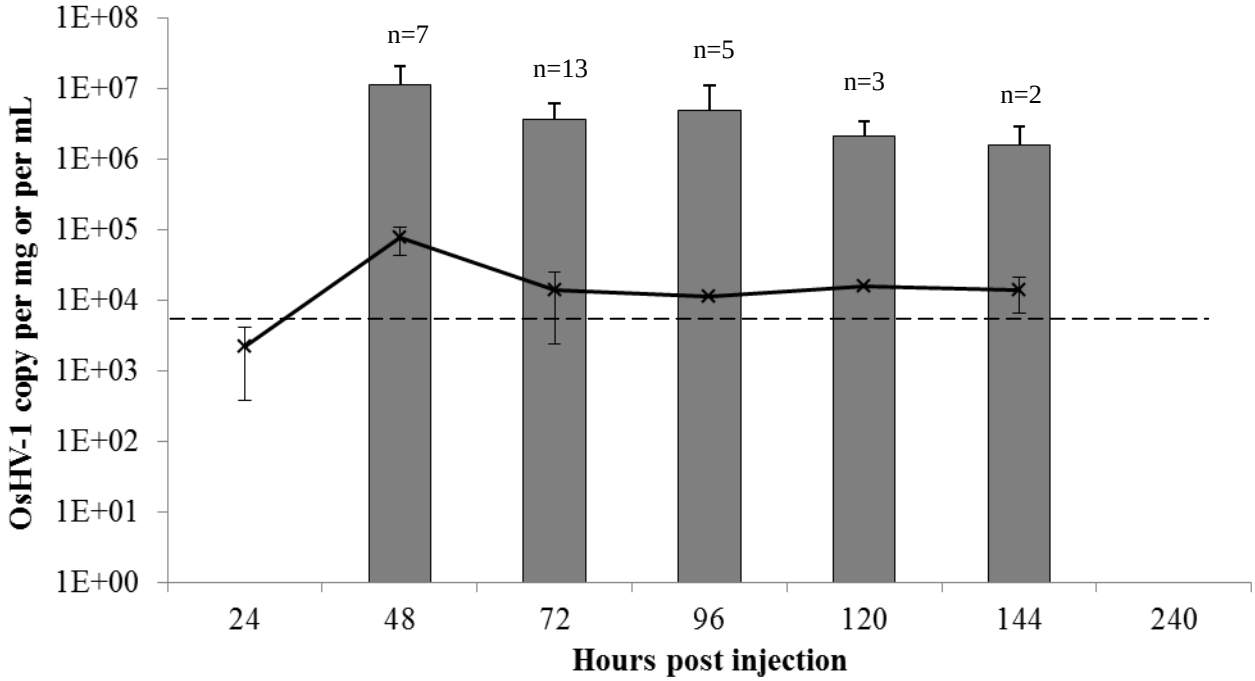

A
sHV-1 copy number per mg or mL
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
n=3
n=4
n=12
n=2
n=1
n=1
n=1
B
HV-1 copy number per mg or mL
n=1
n=7
n=11
n=1
n=1
n=1
n=2
C
sHV-1 copy number per mg or mL
n=4
n=11
n=1
n=3
n=1
n=1
n=1
n=2
D
OsHV-1 copy number per mg or mL
1E+00
n=1
n=1
n=3
n=2
n=5
n=1
n=2
n=2
n=7
24
48
72
88
96
120
144
168
192
210
240
Hours post injection

Figure 4. Viral load in oyster gills (bars), water (unbroken line) and inoculum (dashed line) sampled in experiment 3. The inoculum was kept at +4°C for three months before being injected into naïve oysters. All of the oysters sampled were dead individuals that were systematically removed from the tanks.Results are expressed as copies per mg for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number above each bar represents the number of oysters analyzed at each time.



Tables

Table 1. Hazard ratios and their 95% confidence intervals based on the survival analyses conducted to compare hazard of death for the different treatment groups in experiments 2 and 3.

Treatment groups	Parameter estimate	Standard error	Hazard ratio	95% CI of hazard ratio
Experiment 2				
5 x 10^6 copies per mL	3.47	0.71	32.02	7.9, 129.2
5 x 10^5 copies per mL	3.18	0.74	24.02	5.6, 102.2
5 x 10^4 copies per mL	3.21	0.73	24.82	5.9, 104.3
5 x 10^3 copies per mL	2.29	0.74	9.91	2.3, 41.9
5 x 10^2 copies per mL*	0.00	0.00	1.00	
Experiment 3				
+4°C for 3 months	4.05	0.78	57.33	12.4, 265.0
Room temperature	1.72	0.75	5.56	1.3, 24.1
-20°C	0.71	0.73	2.04	0.5, 8.5
-80°C	0.00	0.00	1.00	

Table 2. Viral concentrations in a fresh inoculum (prepared and analyzed in July 2012) and some aliquots kept at different storage temperature for 3 months (analyzed in October 2012). Results are expressed in OsHV-1 DNA copies per mL.

Fresh inoculum	Room temperature	+4°C	-20°C	-80°C	Liquid nitrogen
1.4 x 10^6	6.4 x 10^2	7.1 x 10^3	3.1 x 10^5	3.1 x 10^5	6.9 x 10^3