

Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian ostreid herpesvirus-1 (OsHV-1) μ Var strain

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ABSTRACT: In Australia, the spread of the ostreid herpesvirus-1 microvariant (OsHV-1 μ Var) threatens the Pacific oyster industry. There is an urgent need to develop an experimental infection model in order to study the pathogenesis of the virus under 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 μ Var strain. Experiments were conducted to test (1) virus infectivity, (2) the dose–response relationship for OsHV-1, and (3) the best conditions in which to store infective viral inoculum. Intramuscular injection of a viral inoculum consistently led to an onset of mortality 48 h post-injection and a final cumulative mortality exceeding 90%, in association with high viral loads (1×10^5 to 3×10^7 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 mo). Storage of purified viral inoculum at $+4^\circ\text{C}$ for 3 mo 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 *Crassostrea gigas* under experimental conditions.

KEY WORDS: Ostreid herpesvirus-1 · *Crassostrea gigas* · Experimental infection model · Dose–response · Inoculum preparation · Storage conditions

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INTRODUCTION

Episodic herpes-like virus infections in Pacific oysters *Crassostrea gigas* have been reported in many European countries (France, the UK, Jersey, Ireland, Spain and The Netherlands) and also in the USA (Renault et al. 1994, Renault & Novoa 2004, Davison et al. 2005, Friedman et al. 2005, Schikorski et al. 2011b). Since 2008, mortality outbreaks have mainly been associated with the presence of a new herpesvirus strain, the ostreid herpesvirus-1 microvariant (OsHV-1 μ Var, hereafter simply 'OsHV-1') infect-

ing Pacific oysters during the summer period, concomitant 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 occurred for the first time in Australia in a small population of commercially farmed triploid Pacific oyster spat in Woolloomare Bay within the Georges River/Botany Bay estuary, New South Wales (NSW) (Jenkins et al. 2013). Epidemiological investigations were conducted in Botany Bay in order to identify critical risk factors leading to mortality (Paul-

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Pont et al. 2013a,b). A little over 2 yr 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 related mass mortalities observed in commercial *C. gigas* leases (Paul-Pont et al. 2014). Due to the threat that OsHV-1 poses to the entire Australian Pacific oyster industry, research activities were first directed towards (1) a mollusc breeding program to develop disease-resistant Pacific oysters and (2) conducting 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 for a detailed study of the virus and the mechanisms underlying infection and death (pathogenicity, host immunity, risk factors). Indeed, important epidemiological features differ between the European and the Australian OsHV-1 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 underlines the need to study the Australian OsHV-1 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 strain purified from naturally infected oysters. Successful infection models using intramuscular (IM) injection that had already been developed in France (Schikorski et al. 2011a,b) were used as a basis to develop a 'local' infection model. Four experimental infection trials were performed in order to test (1) the infectivity of the Australian OsHV-1 strain using infected material sampled from the Georges River during the summer of 2011/2012 (Expts 1a and 1b), the dose–response relationship for OsHV-1 (Expt 2) and the best storage conditions in which to keep purified viral inoculums (Expt 3). Daily mortality was recorded for a period of 10 d. Viral concentrations in oyster tissues and seawater were also measured by real-time quantitative PCR during the time course of the experiments.

MATERIALS AND METHODS

Experiments

A total of 4 experiments were sequentially performed. In Expts 1a and 1b, the aim was to assess whether the disease was reproducible in laboratory conditions using the Australian OsHV-1 strain (tem-

porally replicated experiments). In Expt 2, the aim was to determine a dose–response relationship between the quantity of viral particles injected and the cumulative mortality of oysters. The aim of Expt 3 was to identify the appropriate storage conditions required to maintain the viability of the Australian OsHV-1 strain as a purified inoculum under laboratory conditions.

Origin of oysters and acclimation period

All naive oysters used in this study were hatchery single seed triploid oysters (Shellfish Culture) and came from the same batch (11 mo old; 40 to 50 mm in 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, 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 (Expt 1a), August 2012 (Expts 1b and 2) and September 2012 (Expt 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 less than 12 h after sampling. Water temperature was set to correspond to the environmental conditions in the field (15 to 18°C), and salinity was fixed at 30 ppt. The oysters were acclimated for 1 wk 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 d⁻¹ until it reached 22°C (the temperature used during the experiments). Oysters were fed daily by addition of an adequate quantity (4×10^5 to 1.6×10^7 cells ml⁻¹) of shellfish diet 1800 (Instant Algae®, Reed Mariculture) containing a mix of *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii* and *Tetraselmis* sp. No mortality was recorded prior to the experiments.

Inoculum preparation

Oyster tissue homogenate from OsHV-1 infected and non-infected Pacific oysters were used to prepare infected and control inoculums, respectively. OsHV-1 infected Pacific oysters (12 mo old; length: 79.5 ± 4.6 mm, mean \pm SD) were collected from the Georges River, NSW, Australia during a disease outbreak on 24 November 2011. Control oysters (6 mo old; 52.7 ± 8.8 mm) were collected from the Hawkesbury River on 19 December 2011. Both batches of oysters (infected and control) were previously tested by PCR to confirm their respective status (i.e. infected or control) and were stored at -80°C prior to being used for each experiment. For Expts 1 and 2, a total of 5 oysters from each batch were removed from storage at -80°C 1 d 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 to 0.12 g) was sampled from each oyster and stored briefly 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 to 12 g) into a 50 ml sterile tube maintained in wet ice. These tissues were weighed and diluted by adding 10 volumes of $0.22 \mu\text{m}$ filtered synthetic seawater (FSSW) to the tube. Tissues were homogenised using a MiniMix[®] stomaching machine (Crown Scientific) for 1 min at maximum speed. After centrifugation ($1000 \times g$, 5 min, 4°C), the 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, 0.45 and $2 \times 0.22 \mu\text{m}$ pore sizes. The purified homogenate, or inoculum, was maintained on ice prior to injection.

For Expt 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 injection. The concentrations of the infected inoculums were 1.4×10^6 and 1.3×10^7 viral DNA copies ml^{-1} in Expts 1a and 1b, respectively. No viral DNA was detected in the control inoculums.

For Expt 2, the inoculum was freshly prepared and 5 concentrations were tested (undiluted at 5×10^6 viral DNA copies ml^{-1} , and 10-fold serial dilutions: 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) in parallel with a control

group. Accordingly, the undiluted inoculum was diluted 4 times with FSSW before being filtered twice using $0.22 \mu\text{m}$ 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 injection.

For Expt 3, the inoculum that had been freshly prepared in July 2012 (Expt 1) was dispensed into 4 tubes (5 ml) separately to be kept at room temperature (RT), $+4^{\circ}\text{C}$, -20°C , -80°C ; liquid nitrogen was also used. In early October 2012 (+3 mo) the different inoculums were thawed overnight at $+4^{\circ}\text{C}$ (if required) and filtered twice using $0.22 \mu\text{m}$ pore size 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 injection. See Table 2 for the viral DNA concentrations of each inoculum.

Experimental infection

Each IM injection was carried out on the same day as the homogenate preparation, except for Expt 3 in which stored inoculums were used.

The IM injection was carried out using 8 to 10 oysters in each 25 l tank, and was conducted in triplicate ($n = 3$ replicate tanks containing a total of 30 oysters treatment⁻¹ for Expts 1 and 3, and 24 oysters treatment⁻¹ for Expt 2). Oysters were taken out of the water (air temperature 22°C) for 24 h prior to immersion in a magnesium chloride solution (MgCl_2 ; 50 g l^{-1}) in 1:4 seawater:distilled water (v/v) for between 1 and 4 h. Once relaxed, 100 μl of filtered tissue homogenate was injected into the adductor muscle of each oyster. Control and 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, and (2) the water temperature was uniform between tanks as it was controlled by equilibration with the air temperature ($21.7 \pm 1.2^{\circ}\text{C}$, mean \pm SD). Salinity was monitored daily and remained stable (30 ppt). Water quality parameters (ammonia, nitrate, nitrite, pH) were monitored daily. In Expt 1b the water was occasionally changed to maintain water quality in acceptable ranges.

All experiments were conducted in a dedicated Level 2 biocontainment facility at the University of Sydney in accordance with biosecurity regulations and protocols.

Sampling strategy

A water sample was collected daily from each tank for PCR analysis (volume sampled = 4 ml, volume tested by PCR = 200 μ l). Mortality was monitored daily for 10 d and no active sampling was performed on live oysters. Mean (\pm SD) daily mortality was calculated among the 3 replicate tanks. Dead oysters were systematically removed from the tanks during the course of the experiment and a piece of gill and mantle (0.08 to 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.

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

Tissue homogenisation. A piece of gill and mantle (0.08 to 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 s^{-1} for 15 s) (Fastprep System, MP Biosciences). Tissue homogenates were then clarified by centrifugation at $900 \times g$ for 10 min in a microcentrifuge. Clarified tissue homogenates were stored at -80°C until required.

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

Real-time PCR. The detection of OsHV-1 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 mg^{-1} of tissue and the quantification limit at 12 copies mg^{-1} of tissue based on previously published guidelines (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 (C_T 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 manu-

ally 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.

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 are presented. All analyses were conducted using SAS statistical program (SAS Institute); Kaplan-Meier curves were created using the 'ggsurv' function in R v.3.0.1 (R Foundation for Statistical Computing).

For calculation of the mean (\pm SD) viral copies mg^{-1} of tissues, we first confirmed the absence of any significant tank effect (using ANOVA, $p > 0.05$; STATISTICA v.10 for Windows) before considering all replicate oysters, regardless of the tank number.

RESULTS

Mortality

During Expt 1a (July), oysters infected by IM injection of the OsHV-1 suspension were characterized by a sudden mortality reaching 70% at 48 h post-injection (hpi). At 72 hpi, the cumulative mortality was 90% and reached 97% at 96 hpi. Only 1 infected oyster survived and was eventually sacrificed at the end of the experiment (240 hpi) 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 Expt 1b (August), with an onset of mortality 48 hpi; cumulative mortality of 96% was reached at 96 hpi (Fig. 1). All OsHV-1 injected oysters were dead at 144 hpi whereas no mortality was observed in the control group. The only difference in the mortality pattern between the

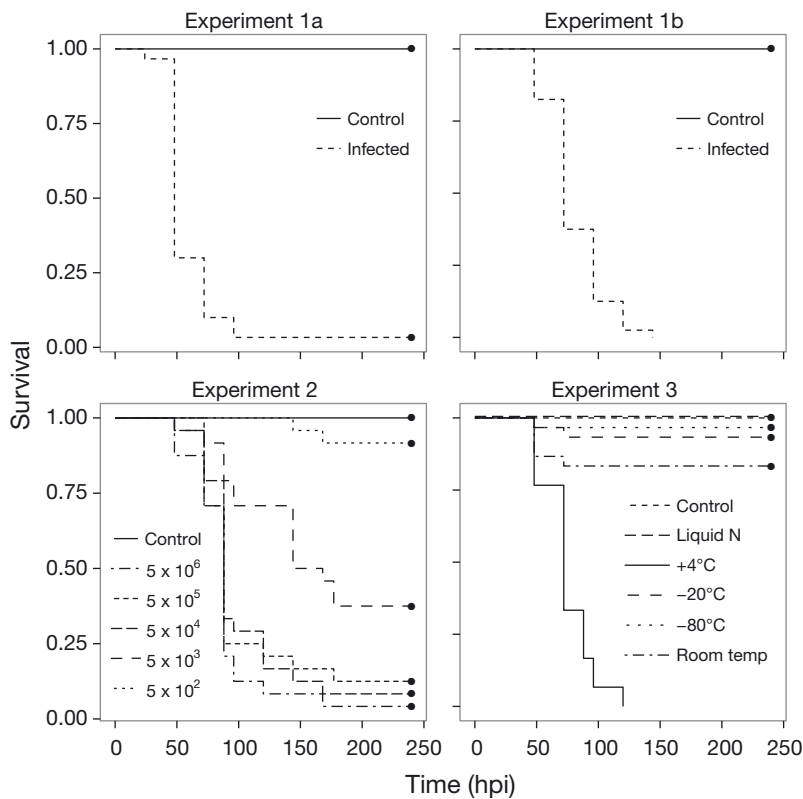


Fig. 1. Survival analyses comparing mortality of Pacific oysters *Crassostrea gigas* exposed to the ostreid herpesvirus-1 microvariant (OsHV-1 μ Var) under different treatment conditions aimed at reproducing the viral infection in laboratory conditions (Expts 1a and 1b), determining dose–response relationships (Expt 2) and storage conditions for the inoculum required to maintain virus viability (Expt 3)

2 experiments concerned the cumulative mortality observed at 48 hpi: 70 % in Expt 1a and only 19 % in Expt 1b (i.e. the onset of mortality was a little slower in Expt 1b). Survival analysis indicated significant differences in survival probabilities between the 2 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 (Fig. 1).

In Expt 2 there was a clear dose-response relationship between the quantity of viral particles injected into the adductor muscle and the survival of oysters (Fig. 1). Mortality was almost nil in the control group (1 oyster died at 72 hpi) and it was therefore not included in the calculation of hazard ratios. Mortality remained low in the highest dilution group (10^{-4})

(2 oysters died at 144 and 168 hpi), while heavy mortalities reaching 88 to 96 % were observed in the groups injected with pure inoculum or the lowest dilutions (10^{-1} and 10^{-2}) (Fig. 1). A 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 ml^{-1} of virus, the other groups (injected with greater quantities of virus) had a greater hazard of death; the hazard was maximum in the group injected with 5×10^6 copies of virus ml^{-1} .

The survival analysis results for Expt 3 are presented in Fig. 1 and Table 1. The injection of the inoculum kept at $+4^\circ\text{C}$ for 3 mo induced significant mortality, similar to the mortality rate observed after injection of the fresh inoculum during Expt 1. Cumulative mortality was 94 % after 96 h and 100 % by the end of the experiment (240 hpi). Survival in the $+4^\circ\text{C}$ group was significantly lower than all other groups, with hazard ratios of 10.31 (95 % CI = 6.0, 17.8) compared to the RT 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 (Fig. 1, Table 1). The hazard of death in

the group injected with the inoculum kept at RT was significantly higher than the groups injected with inoculums kept at -80°C (hazard ratio: 5.56; 95 %

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 Expts 2 and 3

Treatment groups	Parameter estimate	Standard error	Hazard ratio	95 % CI of hazard ratio
Expt 2				
5×10^6 copies ml^{-1}	3.47	0.71	32.02	7.9, 129.2
5×10^5 copies ml^{-1}	3.18	0.74	24.02	5.6, 102.2
5×10^4 copies ml^{-1}	3.21	0.73	24.82	5.9, 104.3
5×10^3 copies ml^{-1}	2.29	0.74	9.91	2.3, 41.9
5×10^2 copies ml^{-1}	0.00	0.00	1.00	
Expt 3				
$+4^\circ\text{C}$ for 3 mo	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	

CI = 1.3, 24.1) and -20°C (2.7; 95% CI = 2.0, 3.8). However, survival was not significantly different between the -20 and -80°C groups (Fig. 1). These last 2 inoculums did not induce any substantial mortality as demonstrated by the cumulative mortalities of 3 and 7% at 240 hpi, respectively. The injection of the inoculum kept in liquid nitrogen did not induce any mortality, nor was any mortality observed in the control group (Fig. 1). Both of these groups were excluded for calculation of hazard ratios.

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 Expt 1a, the viral loads in dead oysters sampled during the first 96 hpi (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 (Fig. 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 (Fig. 2). The only infected survivor was sampled at the end of the experiment (240 hpi) 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 240 hpi 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 48 hpi, respectively, before progressively decreasing to 2.5×10^2 copies ml^{-1} (Fig. 2). For Expt 1b, a different PCR

test (Pepin 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 the water, this did not affect the overall kinetics of infection recorded in oysters (data not shown).

For Expt 2, different concentrations of inoculum were administered ranging from 5.1×10^6 (pure inoculum) to 5.1×10^2 (most diluted inoculum; 10^{-4}) copies ml^{-1} in parallel with 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. In all other conditions (undiluted inoculum, 10^{-1} , 10^{-2} , 10^{-3}), the viral loads in dead oysters sampled during the experiment (i.e. all oysters sampled before 240 hpi) were between 1.1×10^5 and 1.2×10^7 copies mg^{-1} regardless of the concentration of the inoculum used for the injections, except for 1 individual injected with inoculum 10^{-2} (5.1×10^4 copies ml^{-1}) sampled at 168 hpi (viral load in tissue: 2.5×10^4 copies mg^{-1}) (Fig. 3). As in Expt 1a, the viral loads measured in the surviving oysters actively sampled at 240 hpi were much lower than the concentration measured in the dead individuals and in the corresponding inoculum (except for the surviving oysters injected with inoculum 10^{-3}) (Fig. 3).

The viral concentrations in seawater were relatively stable during the first 120 hpi, ranging between 5.1×10^3 and 9.6×10^4 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 (>120 pi) as the tanks were progressively emptied by removing dead individuals.

For Expt 3, the viral concentrations measured in the stored inoculums were systematically lower than the concentration measured in the fresh inoculum in

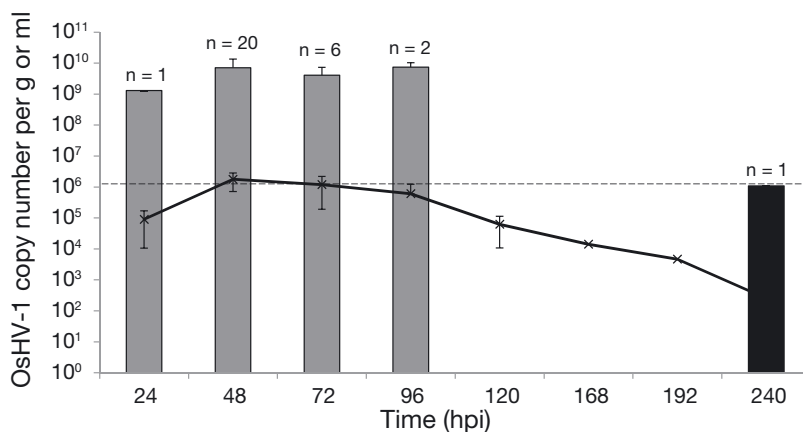
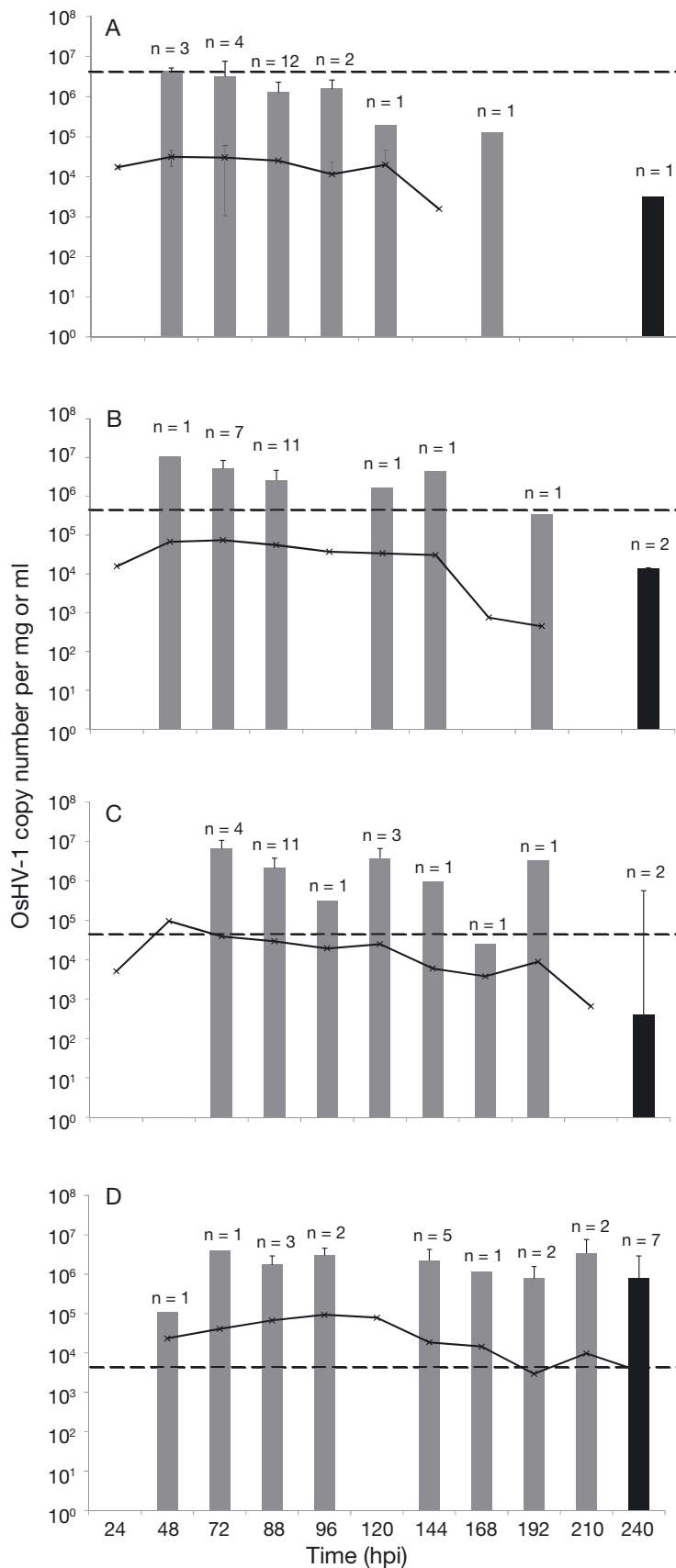


Fig. 2. OsHV-1 loads in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) sampled in Expt 1a. All of the oysters sampled were dead individuals that were systematically removed from the tanks, except for 1 live individual sampled at 240 h post-injection (hpi) (black bar). Results (mean \pm SD) are copies g^{-1} for oyster tissues and copies ml^{-1} for seawater and inoculum. The number above each bar represents the number of oysters analyzed at each time



July 2012 (1.3×10^6 copies ml^{-1}), the lowest concentrations being found in the inoculums kept at RT 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 course of the experiment) or live (sampled at 240 hpi) oysters injected with the viral inoculums kept at -20°C , -80°C or in liquid nitrogen. Among the 5 oysters that died up to 72 h after having been injected with the inoculum kept at RT, 3 were negative for the viral DNA and the 2 remaining oysters demonstrated 1.2×10^1 and 2.4×10^2 copies mg^{-1} (both were sampled at 48 hpi). The only group that demonstrated significant mortalities were the oysters injected with inoculum kept at $+4^\circ\text{C}$ (Fig. 1); viral loads measured in the dead individuals varied between 6.7×10^5 and 3.0×10^7 copies mg^{-1} (Fig. 4). The virus was only detected in the seawater of the tanks corresponding to the $+4^\circ\text{C}$ storage conditions and the concentrations were relatively stable over time, ranging from 2.2×10^3 to 7.7×10^4 copies mg^{-1} (Fig. 4).

DISCUSSION

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

Fig. 3. OsHV-1 load in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) sampled in Expt 2. Concentrations of viral inoculum included (A) undiluted inoculum at 5.1×10^6 , and serial dilutions: (B) 5.1×10^5 , (C) 5.1×10^4 and (D) 5.1×10^3 copies ml^{-1} . All sampled oysters were dead individuals that were systematically removed from the tanks, except for the individuals sampled at 240 h post-injection (hpi) (black bars). Results (mean \pm SD) are copies per mg of oyster tissues; other details as in Fig. 2

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

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

Mortality kinetics

For all experiments, IM injections of pure viral inoculums prepared from Pacific oysters naturally infected by the Australian OsHV-1 μ Var 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 μ Var strain (Schikorski et al. 2011a,b, Segarra et al. 2014). Even though IM injection does not reflect a 'natural' infection mechanism 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 to 5.1×10^6 copies ml⁻¹; Figs. 1–4) with an onset of mortality at 48 hpi and a cumulative mortality exceeding 90% after 7 d of exposure. This result is also in accordance with previous studies using the IM injection method and conducted on 7 to 12 mo old

Pacific oysters (Schikorski et al. 2011a, Segarra et al. 2014). No mortality was observed at 24 hpi, 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 early as 2, 4 and 18 hpi, suggesting that the virus starts replicating in the early hours of the infection (although the first mortalities were not observed until 42 hpi).

Dose–response effects

A dose–response relationship between the concentration of viral particles in the inoculum and the percentage of mortality at 240 hpi was identified through the results of Expt 2. Indeed, at 240 hpi, high cumulative mortalities (>85%) were observed in oysters injected with concentrated inoculums (5.1×10^4 to 5.1×10^6 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) (Fig. 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 2 groups injected with a virulent and a non-virulent inoculum at 2×10^9 and 4×10^6 copies of viral genomes μ l⁻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

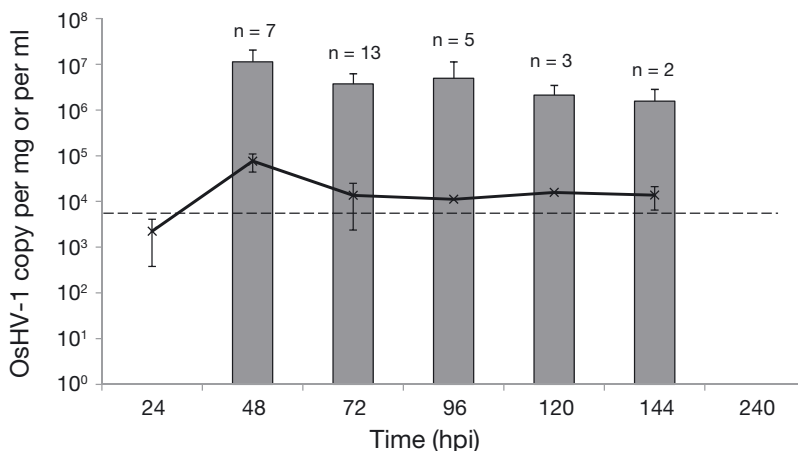


Fig. 4. OsHV-1 load in oyster gills (bars), water (unbroken line) and inoculum (dashed line) sampled in Expt 3. The inoculum was kept at +4°C for 3 mo before being injected into naive oysters. All sampled oysters were dead individuals that were systematically removed from the tanks. Results (mean \pm SD) are copies per mg of oyster tissues; other details as in Fig. 2

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 (5×10^3 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 to be modified in moribund or dead individuals due to degradation processes (Segarra et al. 2014).

Mortality pattern: comparison with other studies

Discrepancies occur in the literature when comparing the initial inoculum concentration and the mortalities obtained at 240 hpi after IM injection: Segarra et al. (2014) observed a cumulative mortality of 63% at 90 hpi after the injection of 1.2×10^5 copies into 7 mo old oysters; Normand et al. (2014) did not observe high mortalities (maximum cumulative mortality of 15% after 7 d of challenge) after the injection of 2×10^9 copies into 8 mo old oysters; and Schikorski et al. (2011a) observed extreme mortality (>90%) after the injection of 1.5×10^7 copies into 12 mo old oysters, which is in accordance with the results of the first 2 experiments presented in this paper. This variability in the short-term mortality kinetics after IM 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 (1) the variation in the natural host due to the utilization of different oyster batches for each experiment; (2) the experimental parameters such as water renewal, density of infected host and nutrition; and (3) the variation in the viral inoculum itself (strain, virulence). Natural variation in the resistance to pathogens, 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 to 10 oysters for 25 l of seawater (without food supply) was used, whereas Normand et al. (2014) placed 40 infected oysters in 20 l tanks in a flow-through system with food, and Schikorski et al. (2011a) 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 Expt 3.

Storage conditions

The storage of the viral inoculum at $+4^\circ\text{C}$ for a period of 3 mo 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 (Fig. 1). In contrast, Corbeil et al. (2012a) demonstrated a loss of infectivity over time when the abalone herpesvirus (AbHV) inoculum was held at $+4^\circ\text{C}$. In addition, the authors mentioned that keeping AbHV inoculum in liquid nitrogen prolonged viral infectivity for longer time periods (at least 21 mo) 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 RT, -20°C , -80°C and in liquid nitrogen for 3 mo did not lead to any significant mortality (<14%), regardless of the concentration of viral particles in each inoculum (Fig. 1, Tables 1 & 2). However, as no cryoprotectant was employed here, this experiment should be repeated with additional treatments (i.e. test a range of cryoprotectants and protocols for progressive reductions in temperature). The experiment was purposely conducted without the use of a cryoprotectant to compare with the success of the previous infection trials (Expts 1a and 1b) for which frozen oysters (-80°C for 6 mo) were employed to prepare the viral inoculums. To our knowledge, this 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. 2011a,b, 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 for 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 conse-

quences of importing frozen oysters from infected areas/states/countries to non-infected locations: Is this a possible means for the spread of virus if infected frozen products 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.

OsHV-1 load in oysters tissues

The viral concentrations measured in the gills and mantle of dead oysters collected during each infection trial were high, ranging 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 Expt 2, and 1, 2 and 5 dead oysters from -80°C , -20°C and RT treatments in Expt 3, respectively). This result suggests that regardless of the time since infection and the quantity of viral particles injected into the animal, the quantity of viral particles must reach a threshold in oyster tissues before causing death. This is consistent with previous field and lab studies demonstrating that viral concentrations exceeding 10^6 to 10^7 copies mg^{-1} are systematically associated with dead or dying 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 (240 hpi) demonstrated significantly lower viral concentrations in their tissues (i.e. below 3.1×10^3 copies mg^{-1}), with a few exceptions (2 live oysters in the treatments 10^{-3} and 10^{-1} in Expt 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.

OsHV-1 variation in seawater

During the first 24 h, average viral concentrations in the water increased from 0 (data not shown) to 9.0×10^4 copies ml^{-1} in Expt 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 Expt 2, and 2.2×10^3 copies ml^{-1} in Expt 3 ($+4^\circ\text{C}$ condition) (Figs. 2–4). Regardless of the infection trial or the initial concentration of viral particles in the inoculum, the average

viral concentrations measured in the water remained relatively stable over the first 120 h, 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 (>120 hpi) were likely due to the progressive removal of dead individuals. These results are in accordance with Schikorski et al. (2011a), who demonstrated an increase in viral concentrations in water up to 1×10^5 copies ml^{-1} during the first 48 h following 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.

CONCLUSIONS

The present work has demonstrated that infection trials can be successfully developed in 'local' conditions using the Australian OsHV-1 μVar strain and healthy, virus-free Pacific oysters from a non-infected estuary. However, great precautions must be taken regarding (1) the source for the viral inoculum and its stability over time; (2) the characteristics (age, physiology, genetics, life history) of the oysters used for the infection trials; and (3) the experimental conditions (nutrition, infected host density, water renewal) in order to develop a reproducible and transferable infection model. Conservation of the viral inoculum at $+4^\circ\text{C}$ provided the best outcome with similar cumulative mortality observed in comparison with the results of the first experiment performed 3 mo earlier 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 mo), 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 an unintentional means of translocation of the virus.

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