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Effect of water temperature on mortality of Pacific oysters *Crassostrea gigas* associated with microvariant ostreid herpesvirus 1 (OsHV-1 µVar)

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ABSTRACT: The ostreid herpesvirus 1 microvariant (OsHV-1 µVar) causes mass mortality of Pacific oysters *Crassostrea gigas*. Water temperature can directly influence the incidence of disease or correlate with seasonal changes in the environment and oyster physiology that modify the susceptibility of the oysters to disease. The effect of water temperature was evaluated in controlled laboratory conditions by intramuscular injection of OsHV-1 µVar after acclimation of 8 mo old spat and 17 mo old adult oysters at 4 different temperatures (14, 18, 22 and 26°C). Mortality was 84 and 77% at 26 and 22°C, respectively, compared to 23% at 18°C and nil at 14°C. There was a statistically significant interaction between the dose of OsHV-1 µVar and water temperature. At 18°C, mortality occurred exclusively at a dose of 10^6 OsHV-1 µVar genome copies per oyster whereas at the higher temperatures, oysters challenged with 10^3 copies per oyster also died. Mortality did not occur at 14°C and OsHV-1 µVar was detected in tissues of only 1% of the oysters after 14 d. When accounting for temperature and dose, spat (8 mo) were 2.7 times more likely to die than adults (17 mo). Our study confirms a direct effect of water temperature on infection and disease caused by OsHV-1 µVar. We identified a threshold water temperature of between 14 and 18°C below which productive infection does not occur and the requirement for a higher dose of OsHV-1 µVar to initiate infection at 18°C than at 22°C. These results have implications for predicting and managing disease outbreaks caused by OsHV-1 µVar.

KEY WORDS: *Ostreid herpesvirus 1* · OsHV-1 µVar · Pacific oyster · *Crassostrea gigas* · Water temperature · Laboratory challenge · Dose-response · Disease susceptibility

INTRODUCTION

A microvariant genotype of the species *Ostreid herpesvirus 1* (OsHV-1) was first identified in 2008 in France (Segarra et al. 2010). This pathogen was shown to be associated with recurrent mass mortality events in the Pacific oyster *Crassostrea gigas*, with up to 100% mortality of juvenile oysters (Schikorski et al. 2011b, EFSA 2015). OsHV-1 is a member of the family *Malacoherpesviridae* within the order *Herpesvirales* (Davison et al. 2005, ICTV 2013). The World Organisation for Animal Health (OIE) defines microvariant genotypes of OsHV-1 (OsHV-1 µVar) as those which are characterised by a deletion in the microsatellite locus upstream of open reading frame (ORF) 4, and several polymorphisms compared with the reference genotype (GenBank accession number AY509253) in ORF 4 (C region) and ORF 42/43 encoding an inhibitor of apoptosis. This definition incorporates several variations on the genotype first described as an OsHV-1 microvariant by Segarra et al. (2010).

Disease caused by OsHV-1 µVar has since been identified in several countries including Australia

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(Jenkins et al. 2013), New Zealand (Bingham et al. 2013), Ireland (Peeler et al. 2012, Clegg et al. 2014), The Netherlands (Gittenberger et al. 2016), Italy (Domeneghetti et al. 2014), and Spain (Aranguren et al. 2012, Roque et al. 2012), where it has caused serious disruption or complete cessation of Pacific oyster production (Paul-Pont et al. 2014, EFSA 2015). Additionally, OsHV-1 µVar has been detected in locations where the disease is absent (Dundon et al. 2011, Shimahara et al. 2012, Jee et al. 2013). The incidence of disease is strongly seasonal with occurrence limited from spring to autumn in waterways where OsHV-1 µVar is endemic (Oden et al. 2011, Peeler et al. 2012, Pernet et al. 2012, Paul-Pont et al. 2014, Renault et al. 2014).

Elevation of water temperature during the spring and summer was associated with mass mortality events in Australia, France and Ireland (Pernet et al. 2012, Clegg et al. 2014, Paul-Pont et al. 2014, Renault et al. 2014). A threshold temperature above which mortality occurred has been reported to be ~16°C in France and Ireland (Pernet et al. 2012, Clegg et al. 2014, Renault et al. 2014, Petton et al. 2015). Furthermore, in France a water temperature of 24°C, above which mortalities cease to occur, has been suggested as upper threshold temperature (Pernet et al. 2012). In New South Wales, Australia, disease occurred when the mean water temperature was between 19 and 24°C, although OsHV-1 µVar DNA was present when the water temperature was lower (Paul-Pont et al. 2013a, 2014). In some cases, a sudden change in water temperature by several degrees Celsius rather than reaching a mean threshold temperature has preceded mortality associated with OsHV-1 µVar (Clegg et al. 2014, Renault et al. 2014). However, disease has not always coincided with similar rapid changes in water temperature, suggesting that short- and long-term temperature changes and the presence of the virus are not the only triggers of mortality (Paul-Pont et al. 2013b, Clegg et al. 2014). The temperature of oysters present in the intertidal zone should not be assumed to be equal to the water temperature. As oysters may not be submerged at all times, temperature will also be influenced by the air temperature and solar radiation (Helmuth 1999).

Similar to expression of disease, Petton et al. (2013) found that transmission of the virus most efficiently occurred in the range of 16–22°C and did not occur at 13°C. Other studies showed that an OsVH-1 infected tissue preparation remained infectious for 54 h at 16°C compared to 33 h at 25°C (Martenot et al. 2015) and that OsHV-1 in seawater retained infectivity for 2 d at 20°C (Hick et al. 2016). Previously infected oysters showed high survival, and low quantities of OsHV-1 µVar DNA were detected when held at <14°C, but high mortality and increased quantity of OsHV-1 µVar DNA occurred when these oysters were subsequently exposed to 21°C (Pernet et al. 2015). This might indicate that a subclinical or latent infection with OsHV-1 µVar occurs at low temperature (Pernet et al. 2015, Petton et al. 2015).

While a seasonal increase in water temperature above 16–18°C is associated with recurrent disease outbreaks caused by OsHV-1 µVar, a range of concurrent seasonal changes in the host and environment may be more important in the pathobiology of the disease. Seasonal changes include the availability and type of food and the metabolic changes of the oyster (Soletchnik et al. 2006). The incidence of disease has varied with factors including: seasonal changes in reproductive effort and energy balance (Pernet et al. 2012, 2014); feeding (Evans et al. 2015); and management practices such as farming structures, immersion time and the density of oysters (Pernet et al. 2012, Paul-Pont et al. 2013a, Normand et al. 2014, Petton et al. 2015, Whittington et al. 2015a). Furthermore, the immune function of oysters is altered by water temperature (Green et al. 2014a,b).

Defining the effect of water temperature on disease caused by OsHV-1 µVar is not possible from field observations alone. The aim of this study was to assess the direct effect of water temperature on the outcome of OsHV-1 µVar infection under controlled laboratory conditions. Infection by intramuscular injection enabled administration of a measured dose of OsHV-1 µVar DNA to all oysters at the same time and eliminated variation due to factors that influence virus transmission.

MATERIALS AND METHODS

Oysters

Two batches of triploid Pacific oysters *Crassostrea gigas* were produced in a Tasmanian hatchery and grown under commercial conditions on fixed long lines in the Shoalhaven River, New South Wales, Australia. Each batch was certified to be OsHV-1 µVar free by a government laboratory, and this estuary is considered free of OsHV-1 µVar (Herbert 2011). The 2 batches were transferred to the laboratory in January 2015 at the ages of 8 mo (spat, Batch ID: SPL14B, length 61.6 ± 8.4 mm [mean ± SD], weight 8.5 ± 3.7 g) or 17 mo (adults, Batch ID:

SPL13B, 48.9 ± 6.4 mm, 11.6 ± 4.6 g). The batch of adult oysters had a greater mass but shorter shell length because of a deeper cup shape compared to younger oysters, reflecting previous growing conditions. Thus, rapidly growing young oysters and slower growing older oysters from commercial farming conditions were tested.

Husbandry and acclimation

The experiment was conducted in a temperature-controlled level 2 physical containment aquatic animal facility (University of Sydney, Camden Campus). The air temperature was maintained at 22°C with a 12 h photoperiod for the duration of the trial. The oysters were housed in 15 l plastic tanks, 6 of which were connected in parallel in a 250 l recirculation system; there were 4 separate systems. Each system was attached to an independent bio-filter (Fluval 406) and a UV unit, both of which were removed for 2 h d^{-1} for feeding. The systems contained artificial seawater (ASW) with a salinity of 30 ppt prepared from dechlorinated water and artificial sea salt (Red Sea Salt). Water temperature was maintained for each system using thermostatically controlled aquarium heaters (AquaOne) and in-line chiller units (DBA-110, Daeil). Temperature data loggers (Thermocron) recording the temperature every 30 min were placed in the sump and in tanks 1 and 4 of each system. Salinity, total ammonia nitrogen and pH were measured every second day (API salt water master test kit, Aquarium Pharmaceuticals). Negative control oysters that were injected with an inoculum that was free of OsHV-1 µVar were maintained separately in 5 l of aerated ASW in vessels floated in each sump of each system so as to provide the same water temperature profile for acclimation and during the trial. Exposure to light, concentration of feed and stocking density were similar to that of the challenged oysters without the potential for waterborne exposure to OsHV-1 µVar. Water quality for the negative controls was maintained by exchanging 50% of the ASW each day.

Oysters were fed commercial algal mix *Isochrysis*, *Pavlova*, *Thalassiosira weissflogii* and *Tetraselmis* (Shellfish diet 1800, Reed Mariculture). A maintenance ration was calculated based on the oysters' wet meat weight according to the manufacturer's instructions and corresponded to 12.5 ml d^{-1} for each recirculation system and 1 ml d^{-1} for the controls (Reed Mariculture).

Oysters were purged without feed in ASW overnight and 10 adult (SPL13B) and 10 spat (SPL14B) oysters were randomly assigned to each of the 6 tanks in each recirculation system. A polypropylene mesh divider separated the age groups within each tank. Six oysters of each age were randomly assigned as negative controls at each temperature. The water temperature in each recirculation system was initially 20°C for 2 d, reflecting water temperature in the field, before being adjusted by 1°C d^{-1} until reaching the target temperatures: 14, 18, 22 and 26°C. Oysters were held at their target temperature for at least 48 h prior to inoculation.

OsHV-1 µVar inoculum

A fresh stock of OsHV-1 µVar was prepared immediately prior to the trial by amplification of an OsHV-1 µVar stock in donor oysters (SPL13B, n = 8). The source of the stock virus was oysters from the Georges River (NSW, Australia) that were naturally infected with OsHV-1 µVar in 2011. The the identity of the µVar virus was confirmed by sequencing. A 0.2 µm filtered tissue homogenate was prepared as described by Paul-Pont et al. (2015). The virus stock was archived at −80°C with 10% v/v glycerol and 10% v/v foetal bovine serum (Sigma). The donor oysters were relaxed in 50 g $MgCl_2$ (Sigma) per liter of dechlorinated tap water prior to injection of 100 µl of a 1/100 dilution of the virus stock in sterile ASW into the adductor muscle. The donor oysters were maintained in ASW at 20°C for 4 d without feed.

The entire mantle and gill was excised from the donor oysters and homogenised by stomaching at maximum speed for 2 min (MiniMix, Crown Scientific) with 10 ml ASW. The homogenate was made up to 1/10 w/v with additional sterile ASW and centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was filtered to 0.2 µm using syringe filters (Minisart, Sartorius). The OsHV-1 µVar genome was quantified by qPCR, and the clarified and filtered tissue homogenate was stored at 4°C for 1 d prior to administration. Immediately prior to use the homogenate was diluted to 1 in 50 and 1 in 5000 v/v with 0.2 µm-filtered, sterile ASW to produce inocula with a dose of 10^6 and 10^3 OsHV-1 µVar genome equivalent copies per 100 µl. A negative control inoculum was prepared by the same procedure from a cryopreserved tissue homogenate prepared from oysters that tested negative for OsHV-1 µVar DNA.

Inoculation of oysters with OsHV-1

Oysters were relaxed in a 50 g $MgCl_2$ (Sigma) per liter of dechlorinated water maintained at the temperature of each treatment group. The oysters were challenged by injection of 10^6 or 10^3 OsHV-1 µVar genome equivalent copies per oyster in a total volume of 100 µl into the adductor muscle using a 25 gauge needle and 1 ml syringe. Half of the tanks in each system were assigned the high dose of 10^6 OsHV-1 µVar genome copies and half the low dose of 10^3 OsHV-1 µVar genome copies. The same inoculum was administered to all oysters regardless of size. The negative control homogenate was diluted 1 in 50 w/v with 0.2 µm filtered-sterile ASW prior to injection of 100 µl into the adductor muscle of the control oysters.

Experimental design

For each of the 4 target water temperatures, 1 recirculation system consisting of 6 tanks was maintained. Each tank contained 10 oysters from the adult batch that were physically separated with a mesh divider from 10 oysters from the spat batch. There were 120 oysters at each temperature, half of which were challenged with the higher dose and the other with the lower dose of OsHV-1 µVar. There were 12 negative control oysters for each water temperature, half from each age batch.

Observation and sample collection

Every oyster was inspected every 12 h, and dead oysters were removed and stored at −80°C. Oysters were considered to be dead when they were open, non-responsive to disturbance of the tank and did not retract the mantle following stimulation with a 22 gauge needle. Feeding was monitored by visual observation of the rate at which algae were cleared from the water. A random sample of 20 live oysters was taken at 7 d post challenge to establish the prevalence of the virus in the 14 and 18°C treatment groups. All surviving oysters were sampled at the end of the trial, 14 d post challenge.

Detection and quantification of OsHV-1 µVar

Tissues were processed according to previously described methods (Paul-Pont et al. 2013a, Whittington et al. 2015b). Briefly, a sample of gill and mantle was excised (pooled weight 0.08–0.12 g) and placed in a 2 ml screw cap tube with 1 ml of ultrapure water (Ultrapur) and 0.4 g of 0.1 mm silica zirconia beads (Daintree Scientific). The samples were homogenised using a TissueLyser II (Qiagen) for 120 s; the tubes were inverted for a second 120 s homogenisation cycle and then centrifuged at 900 × *g* for 10 min. The supernatant was stored at −80°C. Nucleic acids were purified from 50 µl of supernatant with the MagMAX-96 Viral Isolation Kit (Ambicon, Life Technologies) and a magnetic particle processor (MagMAX Express 96 Applied Biosystems, Life Technologies) according to manufacturer's directions with the AM1836 deep-well standard program (Ambicon, Life Technologies). Purified nucleic acids were eluted in 75 µl elution buffer and stored at −20°C.

A real-time quantitative PCR (qPCR) assay targeting ORF 99 of the OsHV-1 µVar genome (B region) that encodes an inhibitor of apoptosis was modified from the method described by Martenot et al. (2010). Duplicate 25 µl reactions were prepared with the Ag Path ID One-step RT-PCR kit (Life Technologies), BF and B4 primers (900 nm), OsHV-1B probe (250 nm) and 5 µl of undiluted nucleic acid template from the sample of each individual oyster.

The thermocycling program conducted with a real time PCR system (Mx3000P, Stratagene) was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Each plate of 96 reactions included a 10-fold dilution series of the plasmid pOSHV1-Breg for quantification (Paul-Pont et al. 2013b); duplicate OsHV-1 µVar positive and negative tissue homogenates (extraction controls); and nil template controls. The ROX normalised FAM fluorescence signal was analysed with a propriety algorithm (Stratagene) to identify positive results and estimate the quantity of OsHV-1 µVar DNA in samples in which both replicates had a logarithmic increase above the threshold value. Samples which satisfied the criteria for detection, but produced a threshold cycle (Ct) value greater than the range of the standard curve, were indicated as below limit of quantification (BLOQ) and were not included in quantitative analyses.

Statistical analysis

Survival analyses were conducted according to the method described in Dohoo et al. (2003). The survival time was defined as the number of hours from OsHV-1 µVar exposure to mortality for oysters that tested positive for OsHV-1 µVar DNA by qPCR. Kaplan-

Table 1. Prevalence and quantity of OsHV-1 µVar DNA (range of OsHV-1 µVar genome equivalents mg^{-1} tissue) in apparently healthy Pacific oysters *Crassostrea gigas* at 7 and 14 d post challenge and total mortality after 14 d, with viral concentration detected in oyster tissues at the time of death. Prevalence calculation: at 7 d post challenge, from samples randomly selected from all the apparently healthy oysters in each group; at 14 d post challenge, considering all surviving oysters. BLOQ: below the limit of quantification. For dead oysters, the number of oysters with greater than 10^4 OsHV-1 µVar genome copies mg^{-1} is given in parentheses; a threshold associated with mortality Oden et al. (2011). n/s: not sampled

Water temperature (°C)	Apparently healthy oysters						Dead oysters		
	7 d post challenge			14 d post challenge					
	n	Prevalence (%)	OsHV-1 µVar quantity	n	Prevalence (%)	OsHV-1 µVar quantity	n	Mortality (%)	OsHV-1 µVar quantity
14	20	30.0	BLOQ	109	1.0	BLOQ	1	1.0	0
18	20	65.0	BLOQ – 1.20×10^3	90	38.6	0 – 3.39×10^2	23	22.8	1.12×10^2 – 7.08×10^4 (7)
22	0	n/s	–	34	2.7	1.26×10^3	98	76.9	6.17×10^1 – 6.76×10^5 (79)
26	0	n/s	–	20	10.0	BLOQ	112	83.8	5.37×10^2 – 1.58×10^7 (106)

Meier survival curves were plotted for each set of replicate treatment groups and for each experiment factor. Curves were compared using the Mantel-Cox log-rank test. The replicate treatment groups were not significantly different (all $p > 0.05$), so they were combined for further statistical analysis. Oysters that were alive at the end of the trial were considered to be censored at the time of sampling, regardless of OsHV-1 DNA test status. A Cox proportional hazards (PH) model with shared frailty to account for clustering was fitted for treatment groups at 18, 22 and 26°C using the Breslow method for tied events (STATA statistical software Version 13). A significant interaction between water temperature and dose was identified and these factors could not be separated. The assumptions of the PH model were assessed graphically using log-cumulative hazard plots for each parameter in the model and statistically using the scaled Schoenfeld residuals. The goodness of fit was confirmed by the Nelson-Aalen cumulative hazard and Cox-Snell residue plots. The model was assessed under conditions of complete positive and negative correlation.

The quantities of OsHV-1 µVar DNA were $\log_{10}$ transformed to satisfy the assumption of normality and compared across different treatments using a restricted maximum likelihood, linear mixed model (REML) (GenStat, 16th edition, 2015, VSN International). Water temperature, dose of virus and age of the oyster were considered as fixed effects, with tank as a random effect. Pairwise comparisons of estimated means used the least significant differences, with significance accepted at $p < 0.05$.

RESULTS

The water temperature remained within 1°C of the target temperature for each system after the acclimation period, with total ammonia nitrogen < 0.5 mg l^{-1} and pH 8.2. Mortality did not occur, nor was OsHV-1 µVar DNA detected in the control oysters at any temperature (n = 48). The control oysters were considered to be healthy based on their opening and closing responses throughout the trial.

There was no mortality associated with the OsHV-1 µVar challenge in oysters maintained at 14°C (Table 1), although 1 oyster died at 72 h post challenge and tested negative for OsHV-1 µVar DNA. The total mortality at 18°C was 23% (Table 1), but none of the oysters injected with the lower dose of OsHV-1 µVar died at this temperature. The mortality at 22 and 26°C was 77 and 84%, respectively.

All oysters that died at 18, 22 and 26°C tested positive for OsHV-1 µVar DNA with between 6.17×10^1 and 1.58×10^7 OsHV-1 µVar genome copies per mg of tissue. The quantity of OsHV-1 µVar DNA detected at the time of death was higher than in survivors and was not affected by the initial dose ($p = 0.51$). The quantity of viral DNA at the time of death for oysters at 18°C was approx. 5- and 6-fold lower than at 22 and 26°C, respectively (Fig. 1a). Similarly, accounting for temperature, there was 2.5 times more OsHV-1 µVar DNA detected in the spat batch of oysters compared to the adult batch (Fig. 1b).

There were a low prevalence and low quantities of OsHV-1 µVar DNA in oysters surviving after 14 d (Table 1). A single surviving oyster at 14°C, one at 22°C and 2 survivors at 26°C tested positive whereas 38.6% of 90 surviving oysters at 18°C were positive for OsHV-1 µVar DNA (not shown). The dose strongly influenced the OsHV-1 µVar status of survivors at 18°C; 82% of those receiving the higher dose remained positive compared to 8.2% of those receiving the lower dose. The prevalence of OsHV-1 µVar DNA 7 d after challenge in live oysters at 14°C was 30%, and the quantity of virus was very low. At this

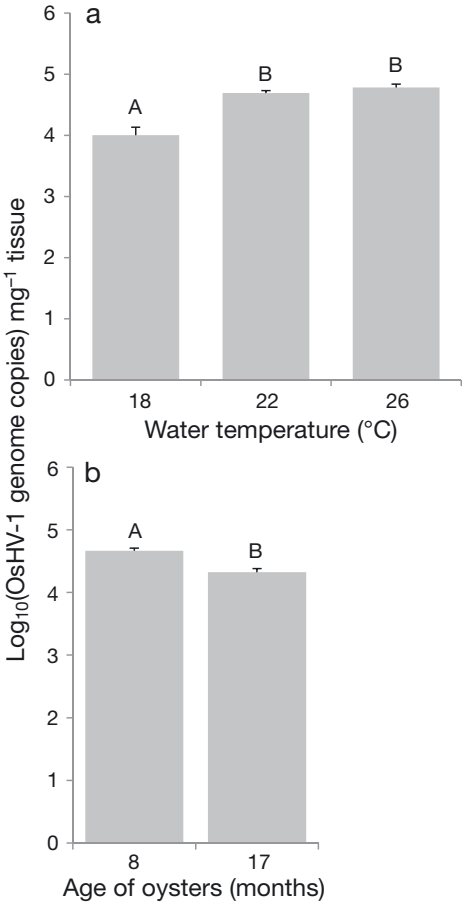

Fig. 1. Quantity (mean + SE) of OsVH-1 µVar DNA (log-transformed no. of OsHV-1 µVar genome copies mg^{-1} tissue) in Pacific oysters *Crassostrea gigas* that died from OsHV-1 µVar challenge at (a) different water temperatures, accounting for age, and (b) different ages, accounting for water temperature. The dose of OsHV-1 µVar did not affect the quantity of viral DNA in the oysters that died (p = 0.51). Groups not sharing uppercase letters are significantly different (p < 0.05, restricted maximum likelihood, linear mixed model)

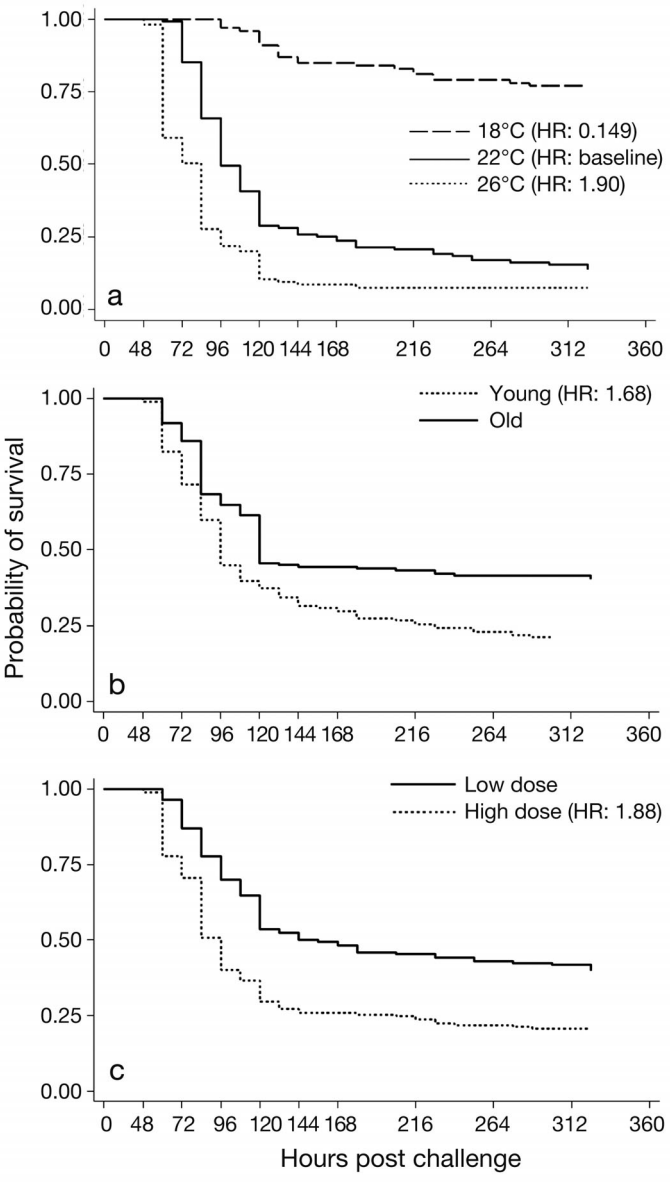

Fig. 2. Kaplan-Meier survival curves and hazard ratio (HR) for Pacific oysters *Crassostrea gigas* challenged with OsHV-1 µVar for each tested experimental factor: (a) water temperature (oysters at 14°C were negative for OsHV-1 µVar and there was a single mortality), (b) age (8 mo old spat vs. 17 mo old adults), and (c) OsHV-1 µVar dose level (low and high dose: 10^3 and 10^6 OsHV-1 µVar genome copies mg^{-1} tissue, respectively)

time, OsHV-1 µVar DNA was detected in 100% of live oysters at 18°C that were administered the higher dose, but in only 33% of the oysters challenged with the low dose (n = 10).

Mortality occurred in a single episode with no secondary peak as might have occurred if there was secondary infection caused by cohabitation with diseased oysters (Fig. 2). Initially, water temperature, age and dose of OsHV-1 µVar were examined in isolation of the other factors, and there was no evidence of any violations of the assumptions of proportional hazards (Fig. 2). On any given day, all oysters held at 26°C were 1.9 times more likely to die with OsHV-1 µVar infection compared to all oysters held at 22°C (i.e. the hazard ratio [HR]; 95% confidence interval [CI]: 1.45–2.50). The median survival time was 84 and 96 h for oysters held at 26 and 22°C, respectively (Fig. 2A). There was a protective effect for oysters at 18°C, with a hazard ratio <1 when compared to oysters held at 22°C (Fig. 2A). Over the experimental period, all oysters from the spat batch were 1.68 (1.29–2.18, 95% CI) times more likely to die with high quantities of OsHV-1 µVar DNA compared to

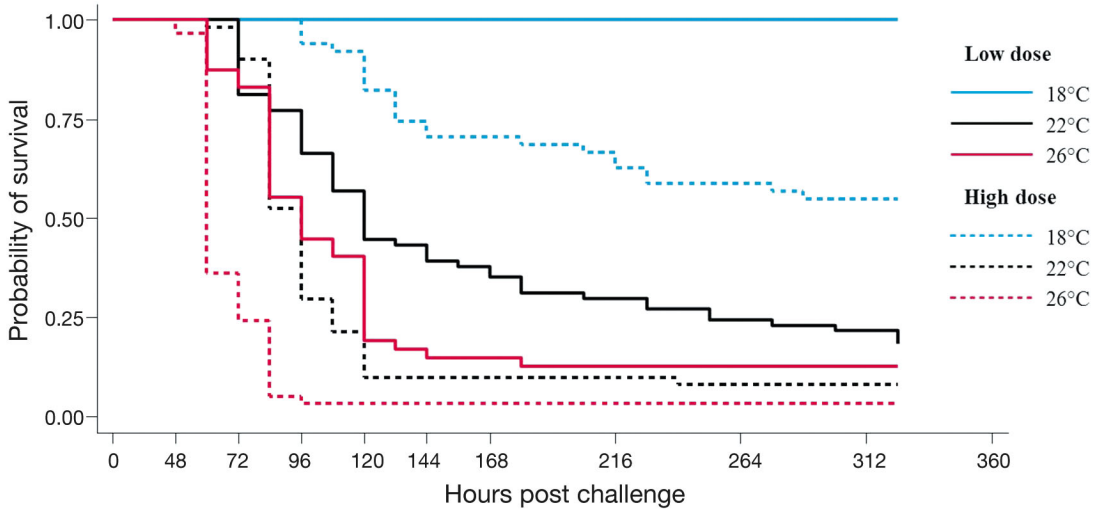

Fig. 3. Kaplan-Meier survival curves for Pacific oysters *Crassostrea gigas* challenged with OsHV-1 µVar showing the interaction between water temperature (18, 22, and 26°C) and dose (10^3 and 10^6 genome copies mg^{-1} tissue)

the older oysters (Fig. 2B). There was nearly twice the risk (HR 1.88; 1.45–2.44 95% CI) of dying with OsHV-1 µVar infection in all oysters given the high dose of OsHV-1 µVar compared to the low dose exposure (Fig. 2C). The median survival time for all oysters given the high dose of OsHV-1 µVar was 96 h compared to 144 h for the low dose.

The Cox PH model enabled a multivariable analysis. There was a significant interaction between water temperature and dose, indicating that the effect of water temperature was different depending on the challenge dose. The factors could not be separated and were considered as 6 separate treatment groups (Fig. 3). The oysters at 14°C were excluded from the PH model because mortality related to OsHV-1 µVar infection did not occur. The final model included the treatment groups shown in Table 2 and was significant ($p < 0.001$). On any given day, oysters held at 26°C were 2.1 and 3.6 times more likely to die with OsHV-1 µVar infection compared to oysters at 22°C when challenged with low and high doses, respectively (Table 2). On any given day, oysters given a high dose of OsHV-1 were 2.1 and 3.8 times more likely to die with OsHV-1 µVar infection compared to oysters challenged with the lower dose when held at 22 and 26°C, respectively (Table 2). There was a protective effect for oysters at 18°C compared to the higher temperatures (Fig. 3, Table 2). The assumption of proportional hazards was not violated for age, as it did not interact with either water temperature or dose of OsHV-1 µVar (all $p > 0.1$). Accounting for water temperature and dose, the young oysters were 2.7 (2.1–3.6 95% CI) times more likely to succumb to OsHV-1 µVar infection compared to older oysters (Table 2). Residual analysis determined the PH model fit to the data and did not detect a significant effect when considered under conditions of complete positive and complete negative censoring.

Table 2. Hazard ratios for Pacific oysters *Crassostrea gigas* challenged with OsHV-1 µVar for each tested experimental factor (temperatures 18, 22, 26°C; doses 10^3 [low] and 10^6 [high] OsHV-1 µVar copies per oyster; age [8 mo old spat]). There was a significant interaction between water temperature and dose of OsHV-1 µVar. –: no mortality observed for the low dose at 18°C

Factor	Level	Hazard ratio	
		Point estimate	95% CI
Low dose	18 vs. 22°C	–	–
	26 vs. 22°C	2.1	1.5–2.9
High dose	18 vs. 22°C	0.2	0.1–0.2
	26 vs. 22°C	3.6	2.7–4.9
22°C	High vs. low dose	2.1	1.7–2.7
26°C	High vs. low dose	3.8	2.5–5.8
Age	Spat (8 mo)	2.7	2.1–3.6

DISCUSSION

This study demonstrates the important direct effect of water temperature on the expression of disease caused by OsHV-1 µVar when administered by injection into the adductor muscle under controlled laboratory conditions. The intramuscular injection challenge method was first described by Schikorski et al. (2011b) in France and developed further in Australia by Paul-Pont et al. (2015). This allowed pathogenesis

to be examined separately from factors that affect transmission and bypasses the external defences of the oyster. The quantity of OsHV-1 µVar DNA in the inoculum administered to each oyster was measured, and the infection was not dependent on the transmission of variable quantities of virus present in the environment or shed by donor animals, as is the case with cohabitation models (Schikorski et al. 2011a, Petton et al. 2013, Evans et al. 2015). Unlike field studies, the laboratory environment enabled water temperature to be evaluated *per se*, with control of other factors that are also seasonally variable such as photoperiod and feed availability. The infection model did not introduce variability in factors other than controlled OsHV-1 µVar exposure that might have influenced pathogenesis, such as co-infection with *Vibrio* spp. (Petton et al. 2013, 2015). Our experiment used 2 batches of oysters to demonstrate a marked and direct effect of water temperature on the outcome of challenge with OsHV-1 µVar. The results are consistent with effects of water temperatures observed during disease in field conditions (Paul-Pont et al. 2014, Whittington et al. 2015a). Further work is required to replicate the experiment, test temperatures between 14 and 18°C in more detail and evaluate oysters with different genetic and life histories.

This study supports the observations of others who have shown a strong association between the onset of mortality and water temperature that is associated with warmer seasons (Petton et al. 2013, Clegg et al. 2014, Paul-Pont et al. 2014, Renault et al. 2014, Pernet et al. 2015). In the present study, mortality related to challenge with OsHV-1 µVar did not occur in oysters at 14°C. OsHV-1 µVar infection may not have been able to establish at this temperature because the virus could not replicate or was cleared by an innate immune response. Alternatively, a subclinical infection may have been established that was not detected by qPCR (Pernet et al. 2015). The risk of disease in these oysters if water temperature subsequently increased was not determined. However, at 18°C, OsHV-1 µVar DNA persisted for at least 14 d in oysters that had been given the low dose, suggesting that viral replication may have occurred without inducing mortality. The incubation period for OsHV-1 µVar-associated mortality in naïve oysters exposed to infected oysters from the field at 17.5°C was 6 d (Petton et al. 2013). A procedure for demonstrating latent infection that involves warming oysters to 21°C has been reported (Pernet et al. 2015, Petton et al. 2015).

In this trial, mortality associated with OsHV-1 µVar replication, as indicated by increased quantities of viral DNA at the time of death, occurred at 18°C but not at 14°C in conditions that were otherwise equal. A strong, direct effect of water temperature on the host–pathogen interaction was indicated, with increased mortality at higher water temperatures. The quantity of viral DNA that was detected in oysters at the time of death was higher with each increase in temperature from 18 to 26°C. The disease outcome might also indicate a deleterious increase in the immune response to the virus that is promoted by the higher temperature (Green et al. 2014a). The effect of both water temperature and the rate of temperature change on the immune responses of the oyster, and the transcriptional changes in the virus genome present opportunities for further research.

In the present study, the threshold dose of OsHV-1 µVar for disease expression was temperature dependent, and for a given dose, total mortality was higher at higher water temperatures. A clear implication of these results is that the dose of virus is likely to be a very important risk factor for an outbreak of disease caused by OsHV-1 µVar. There is an apparent difference between the threshold temperatures at which OsHV-1 µVar-associated mortalities occur in France (16°C) compared to Australia (21°C) (Pernet et al. 2012, Jenkins et al. 2013, Paul-Pont et al. 2014, Renault et al. 2014). It is possible that this difference is due to virus strain differences or differences in oyster genetics between Europe and Australia. Australian and French OsHV-1 µVar isolates are closely related on available genetic sequence; however, comparative analysis has focused on phylogenetic similarities and not virulence traits (Jenkins et al. 2013). Alternatively, the differences might reflect different methods for measuring and reporting water temperature. There can be considerable diel variation in water temperature in the shallow near-shore waters where oysters are grown (Kaplan et al. 2003, Lucas et al. 2006). Thus, an average water temperature obtained in a main channel might not be completely informative.

The risk for OsHV-1 µVar epidemics developing in aquaculture will increase with temperatures greater than 18°C due to a lower lethal dose of OsHV-1 µVar and a higher replication rate of the virus. Thus, both the water temperature and the amount of OsHV-1 µVar present will influence the time of the first outbreak of the season. The dose effect also offers a possible explanation for the sometimes high variation in mortality of oysters of the same age in disease outbreaks in the field (Paul-Pont et al. 2013a, 2014, Clegg et al. 2014). While the disease risk will be influenced by many factors, including those that affect transmission, the importance of dose and tem-

perature was highlighted by this laboratory investigation, which controlled for extrinsic factors that complicate field observations. Disease mitigation strategies have been proposed based on field observations of factors that influence disease (Petton et al. 2015, Whittington et al. 2015a). Improved disease management and prediction of epidemics can be achieved based on better knowledge of the direct influence of water temperature and initial quantity of OsHV-1 µVar on pathogenesis and total mortality.

The environments where oysters are cultivated, including the hydrodynamics of the bay or estuary, are suspected to play a major role in the transmission of the virus through water connectivity (Pernet et al. 2012, Paul-Pont et al. 2013b). Hydrodynamics also influence the local water temperature in the areas where oysters are grown. Generally, these are relatively shallow, likely to be warmed by the sun and subjected to greater temperature fluctuation than the deeper areas that are well mixed by currents (Pernet et al. 2012). Oysters grown on long-lines or in trays in the intertidal zone experience time out of water at low tide and may therefore be relatively heated or cooled compared to the ambient water temperature during this time (Paul-Pont et al. 2013a). The observations of oysters immersed at a static temperature may not provide a complete indication of the disease outcomes expected in cultivated oysters subject to the fluctuations of an inter-tidal environment.

CONCLUSION

This study confirms that water temperature directly influences mortality caused by OsHV-1 µVar infection of oysters, consistent with the pattern of recurrent seasonal disease outbreaks. Higher water temperatures, in the range of 18–26°C probably increase the risk of epidemics associated with OsHV-1 µVar due to a lower lethal dose and a higher viral replication rate. Mortality of oysters injected with OsHV-1 µVar in laboratory conditions depended on the temperature of the water when other factors were controlled. Mortality did not occur and there was no evidence of viral replication at 14°C. Yet the same challenge resulted in mortality exceeding 75% with a high rate of viral replication at 22 and 26°C. The influence of the dose of OsHV-1 was evident at the intermediate temperature of 18°C, where the lower dose did not result in mortality and the higher dose resulted in less severe disease compared to that which occurred at higher temperatures.

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