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Temperature modulate disease susceptibility of the Pacific oyster *Crassostrea gigas* and virulence of the Ostreid herpesvirus type 1

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Abstract :

Temperature triggers marine diseases by changing host susceptibility and pathogen virulence. Oyster mortalities associated with the Ostreid herpesvirus type 1 (OsHV-1) have occurred seasonally in Europe when the seawater temperature range reaches 16–24 °C. Here we assess how temperature modulates oyster susceptibility to OsHV-1 and pathogen virulence. Oysters were injected with OsHV-1 suspension incubated at 21 °C, 26 °C and 29 °C and were placed in cohabitation with healthy oysters (recipients) at these three temperatures according to a fractional factorial design. Survival was followed for 14 d and recipients were sampled for OsHV-1 DNA quantification and viral gene expression. The oysters were all subsequently placed at 21 °C to evaluate the potential for virus reactivation, before being transferred to oyster farms to evaluate their long-term susceptibility to the disease. Survival of recipients at 29 °C (86%) was higher than at 21 °C (52%) and 26 °C (43%). High temperature (29 °C) decreased the susceptibility of oysters to OsHV-1 without altering virus infectivity and virulence. At 26 °C, the virulence of OsHV-1 was enhanced. Differences in survival persisted when the recipients were all placed at 21 °C, suggesting that OsHV-1 did not reactivate. Additional oyster mortality followed the field transfer, but the overall survival of oysters infected at 29 °C remained higher.

Highlights

► High temperature (29 °C) decreases the susceptibility of oysters to the virus. ► There was no alteration of its infectivity and virulence. ► At 26 °C, the pathogen virulence was enhanced. ► Disease mitigation measures based on temperature are discussed.

Keywords : Bivalve, Health, Marine disease, Mortality risk, Temperature, Virulence

Introduction

The risk of disease outbreaks in the marine environment are governed by interactions between host, parasite, and environmental factors [1]. Of all the environmental factors, seawater temperature plays a decisive role in triggering diseases [2]. Temperature modulates the aptitude of a parasite to colonize its host and the ability of the host to defend itself. For example, mass mortalities of shrimps and sea-stars caused by pathogenic viruses occurred during the warm season [3–5]. Similarly, when abalones were exposed to the pathogenic bacteria *Vibrio harveyi*, an increase of seawater temperature of one degree during the spawning season altered the host–parasite relation in favor of the pathogen and lead to epidemic disease [6].

In warm-blooded species, fever, which consists of an increase in body temperature, makes it possible to fight against viral and bacterial infections. Although marine animals are mostly ectotherms (cold blooded), the application of high temperature treatments has proven efficient to treat diseases. For instance, shrimps infected with the white spot syndrome virus at 32°C showed no mortality, whilst they all died at 25°C [3,7].

There is a range of possible influences of high temperature effects on the infectivity of parasites (replication, virulence) and host susceptibility. For instance, high temperature improves shrimp resistance without altering the infectivity of the white spot syndrome virus [3,8]. However, the stability of some shellfish pathogens is altered at high temperature, as reported for the abalone herpesvirus [9].

This study focuses on the effect of high temperatures on the interaction between the Pacific oyster *Crassostrea gigas* and the Ostreid herpesvirus type 1 (OsHV-1). Indeed, OsHV-1 outbreaks have caused mass mortalities in young oysters along the European coastline since 2008

and the virus has spread to Australia and New Zealand [10–13]. Seawater temperature defines the start and the end of OsHV-1 epizootics. In France, the optimal seawater temperature range for disease transmission and subsequent mortalities is between 16°C and 24°C in the field [14,15]. In the laboratory, oyster survival of individuals acclimated to temperatures between 13°C and 29°C exposed to OsHV-1 were much higher when temperatures were exceeded 26°C [16]. An Australian study comparing survival of oysters injected with OsHV-1 at 14°C, 18°C, 22°C and 26°C shows that it was the lowest at 26°C [17]. Furthermore, the infectivity of OsHV-1 kept for 54 h at 25°C is lower than at 16°C [18].

Although the temperature clearly influences the risk of oyster mortality caused by OsHV-1, it is not known at this time whether the temperature affects the host, by modifying its susceptibility to the virus, the pathogen, by acting on virulence, or both. Also, we do not know if oysters that have survived a high temperature infection are able to transmit the virus to healthy animals and if they remain protected against a second infection. The answer to these questions is crucial to develop OsHV-1 control measures from heat treatment.

Here we tested the effect of three temperatures on disease susceptibility of oysters and virulence of OsHV-1. The control was 21°C as this temperature is permissive to OsHV-1 replication and close to the optimal temperature for oyster growth and reproduction [19]. The tested temperatures were 26°C, a temperature whose effects on the survival of the oyster remains to be clarified, and 29°C, a temperature at which the survival of oysters exposed to the virus is improved [16,17]. These two temperatures are in the thermal range of the oyster since the filtration stops only at 33°C and mortality occurs at 38°C [19] (*Petton com pers*).

The first experiment was designed to investigate the effects of high temperatures on OsHV-1 transmission, infection and mortality of oysters. Specific-pathogen-free (SPF) oysters were

injected with OsHV-1 suspension (pathogen donors) or synthetic seawater (controls) and placed in cohabitation with SPF oysters hereafter called “pathogen recipients” or “control recipients” respectively (Fig. 1). These recipients were previously acclimated or directly dipped (not-acclimated) at 21°C, 26°C or 29°C. Their survival was followed for 14 d and the infectivity of OsHV-1 was evaluated (experiment 1A). Next, the recipients were all placed at 21°C, and new SPF oysters were added to the tanks in order to evaluate the potential for virus reactivation and transmission (experiment 1B). Finally, recipients were transferred to a farming area where OsHV-1 induced mortalities were occurring to evaluate the long-term susceptibility to the disease (experiment 1C). Concomitantly, a second experiment (experiment 2) evaluated the effect of temperature on the OsHV-1 suspension *per se*. The viral suspension and synthetic seawater (control) were incubated at 21°C, 26°C and 29°C prior injection in oysters at 21°C. Survival of recipients cohabited with injected oysters was followed for 14 d.

Experimental procedures

Animals and maintenance

Two cohorts of specific-pathogen-free (SPF) oysters were produced under controlled conditions [20,21]. Briefly, wild oysters were collected in Fouras (Marennes-Oleron, France; 46° 00’ 43.2’ ’N, 1° 07’ 02.9’’ W) in August 2015 and were transferred to the Ifremer facilities in Argenton (Brittany, France; 48°34’30’’N, 4°36’18’’ W) for conditioning. These animals were held in 500 L flow through tanks with seawater kept at a constant temperature of 17°C that was enriched with a phytoplankton mixture. Seawater was UV treated and filtered thought 1 µm mesh. Fertilization was performed by stripping the gonads from 100 individuals (1/3 males, 2/3 females) on 23 August 2015 (cohort 1) and 23 February 2016 (cohort 2). The fertilization rate was up to 90%.

The embryos developed in 150l tanks at 21°C for 48h, and D-larvae were transferred to flow-through rearing systems at 25°C. After 13 days, competent larvae were collected and allowed to settle in downwellers. On 1st October 2015 and 1st April 2016, oysters were transferred to Ifremer facilities in Bouin for nursery where were seawater was UV treated and filtered thought 1 µm mesh (Vendée, France, 46°57′15.5″N 2°02′40.9″W).

The two cohorts of SPF oysters were moved back to Argenton on 28 April 2016 and split into five 500 L tanks at 21°C prior to starting the experiments in open flow systems. At this time, oysters from the first cohort were 8 months old, with a mean weight of 1.48 g. Oysters from the second cohort were 2 ½ months old with a mean weight of 0.80 g. The oysters were screened for the herpesvirus by qPCR at all the different production steps it was always undetected [22]. They were fed with a mixture of *Chaetoceros muelleri* (CCAP 1010/3) and *Tisochrysis lutea* (CCAP 927/14) (1:1 in dry weight). Food concentration was set at $1500 \mu m^3.\mu l^{-1}$ of microalgae at the outlet pipe of the tank so that oysters were fed *ad libitum* [23]. Temperature, salinity, pH and oxygen were controlled daily with the WTW probes xi3101, cond340, pH3310 and FDO 925, respectively.

Oysters from the first cohort were either injected with a suspension of OsHV-1 (pathogen donors) or synthetic seawater (controls), or they were not injected and used as recipients in both experiments 1 and 2. Oysters from the second cohort served as new recipients in experiment 1B.

Experimental design

Acclimation of oysters

On 9 May 2016, oysters for injection (N=3000 individuals) were either left at 21°C (control temperature), or gradually increased to 26°C and 29°C at 2°C day^{-1} in three 500 L tanks (one for

each temperature, Fig. S1). At the same time, some of the recipient oysters (N=3600 individuals) were transferred to the experimental room, distributed into twenty-five 45 L tanks and either left at 21°C or gradually increased to 26°C or 29°C (15 tanks were set at 21°C, 5 tanks at 26°C and 5 tanks at 29°C, see Table 1). The remaining recipients were left undisturbed in 500 L tank at 21°C and later served as non-acclimated recipients. Acclimation lasted for 11 days up until 19 May.

Each tank was equipped with a thermostat and heating resistor (Biotherm Ecco Hobby and SCHEIGO Titane 300W, Europrix, France) to maintain the seawater at the desired temperature (Fig. S2). Three tanks (one for each temperature) were equipped with a high-frequency temperature data logger (iButton DS1922L) with 0.1°C resolution and a final accuracy of ± 0.3°C. A light bubbling and a circulation pump (Aquapower 200 superfish 200L/H, Europrix, France) were added in each 45 L tanks to maintain >90% oxygen saturation and seawater homogenization. Seawater salinity was 35‰. The oyster filtration rate was measured daily in each tank as reported in [23] (Fig. S3). The water flow was set at 70 L h^{-1} in the 500 L tanks and 12 L h^{-1} in the 45 L tank. Throughout this time, there was no oyster mortality.

Infection

On 19 May 2016, oysters for injection were myorelaxed in $MgCl_2$ solution at their respective acclimation temperature until valve opening [24]. Pathogen donors were injected with 100µl of viral suspension containing 6.9×10^6 copies of OsHV-1 µVar in the adductor muscle, while controls were injected with the same volume of sterile synthetic seawater. Donors and controls were further incubated for 5 hours at their respective acclimation temperature.

The viral suspension was obtained from 10 infected oysters. The gills and mantle of these oysters were placed in a sterile 50 ml tube containing 10 volumes of artificial seawater (ASW, 9 mL g^{-1}

tissue). Tissues were ground on ice using an Ultraturax (3 × 5 s) mixer. After centrifugation (1000 g, 5 min, 4°C), the supernatant was transferred to a new tube and diluted in 4 volumes of ASW. The homogenate was filtered under sterile conditions using syringe filters at pore sizes of 5 µm, 2 µm, 0.45 µm and 0.22 µm (Millipore, Billerica, USA).

Effect of high temperatures on OsHV-1 transmission, infection, and mortality of oysters (experiment 1-part A)

Injected oysters (pathogen donors and controls) were transferred into the 45 L tanks to cohabit with the recipients acclimated and maintained at 21°C, 26°C and 29°C. Donors were distributed in triplicate tanks while controls were in duplicates for each temperature (Table 1). At the same time, the non-acclimated recipients left at 21°C were added to the tanks that contained pathogen donors or controls, and the volume of water was reduced to 30 L and left stagnant for 12 hours. Dead donors were removed 24 h post-injection (hpi) and all the donors were removed 72 hpi. The biomass of controls and pathogen donors was 230 g while that of recipients was 350 g (Fig. 1).

Survival of acclimated and non-acclimated recipients was monitored for 14 days. Thirty living recipients (both acclimated and non-acclimated, 15 individuals for each group) were sampled in each tank at 0, 12, 24, 48 and 96 h post-cohabitation (hpc). Whole tissues of 9 recipients out of 15 were removed from the shells, pooled together, flash frozen and stored in liquid nitrogen. Then, oyster tissues were crushed in liquid nitrogen with a MM400 homogenizer (Retsch, Eragny, France) and divided for OsHV-1 DNA and viral gene expression.

Effect of previous temperature treatments on survival of recipients at 21°C (experiment 1-part B).

On 2 June 2016, the temperature of all recipient tanks was set at 21°C and new SPF oysters were added. Recipient and new SPF survival was monitored for 11 days up until 13 June.

Effect of previous temperature treatments on susceptibility of oysters to a second infection in a farming area (experiment 1-part C).

On 13 June 2016, the recipients surviving the experiment 1A and B were transferred into the Bay of Brest at Pointe du Chateau in (48° 20' 06.19" N, 4° 19' 06.37" W) where mass mortalities of oysters caused by OsHV-1 were occurring [25], and their survival was followed for 302 days.

Effect of incubation temperature on the OsHV-1 suspension (experiment 2).

On 19 May 2016, the viral suspension and the synthetic seawater were incubated at 21°C, 26°C and 29°C for 5 hours before injecting 100 µl into the adductor muscle of both pathogen donors and controls kept at 21°C. Virus suspension remain infectious for 48 h in seawater at 20°C but infectivity decreases after 24 h [26]. These injected animals remained for 5 h in six separate tanks (one for each combination of injection type and incubation temperature). Injected oysters (pathogen donors and controls) were transferred to the 45 L tanks to cohabit with the recipients acclimated at 21°C. Donors were distributed in triplicate tanks while controls were in duplicates for each temperature (Table 1). The volume of water was reduced to 30 L and left stagnant for 12 hours. Dead donors were removed 24 hpi and all the donors were removed 72 hpi. The biomass of controls and pathogen donors was 230 g while that of recipients was 350 g (Fig. 1). Controls and pathogen donors at 21°C were common to experiments 1 and 2 (Table 1).

OsHV-1 DNA quantification

Level of OsHV-1 DNA was quantified in both control and pathogen recipients, acclimated or non-acclimated, sampled at 0, 12, 24, 48 and 96 hpc in experiments 1A and 2. These analyses were conducted by the Laboratoire Departemental Veterinaire de l'Herault (Montpellier, France) using oyster powder homogenized in sterile artificial seawater [27]. Total DNA was then

182 extracted with a QIAamp tissue mini kit (Qiagen) according to the manufacturer's protocol. The
183 extract was stored at −20°C before detection and quantification according to a real-time PCR
184 protocol based on SYBR Green chemistry [27] with specific primers validated by [28]. The
185 results were expressed as the log of OsHV-1 DNA copies per mg of wet tissue.

186 Viral gene expression

187 Viral gene expression was quantified in acclimated pathogen recipients sampled at 24 and 48 hpc
188 in experiments 1A and 2.

189 *Total RNA extraction and cDNA synthesis.*

190 Total RNA was isolated using Extrac-all (Eurobio, Courtaboeuf, France) at a concentration of 1.5
191 mL 30 mg^{-1} powder, and treated with DNAse I (Sigma, 1 U μg^{-1} total RNA). Samples were then
192 treated with DNAse (DNase Max™ Kit, MO BIO laboratories, Inc) using (1 U μg^{-1} total RNA)
193 to remove genomic DNA. Quality of RNA and quantity were determined using a NanoDrop 2000
194 (Thermo Scientific). First strand cDNA synthesis was performed using the iScript™ cDNA
195 Synthesis Kit (BIO-RAD) with 1µg of RNA [29]. A no reverse transcription was performed after
196 each DNAse treatment using real time PCR to control for the absence of oyster and virus
197 genomic DNA.

198 *Real time PCRs and relative expression.*

199 Six viral genes (ORFs 27, 38, 41, 67, 87 and 99) were selected among the 39 ORFs described by
200 [30]. These ORFs encoded for different protein functions and expressed differently during an
201 OsHV-1 replication cycle [30].

202 The real-time PCR assay was performed in triplicate with 5µl cDNA (1/10 dilution) in a total
203 volume of 10µL. The concentrations of the reaction components were as follows: 10µM of each
204 primer, 1.5µl H2O, 7.5µl of iQ™ SYBR Green Supermix (BIO-RAD). Real time PCR cycling
205 conditions were as follows: activation at 95°C for 5min followed by 45 cycles of 30s at 95°C, 1
206 min at 60°C, and a melting curve program from 95 to 70°C by decreasing the temperature by
207 0.5°C every 10s. Each run included a positive cDNA control (a pool of the 30 cDNA samples of
208 the present experiment analyzed in each amplification plate) and in all cases negative controls
209 (without cDNA) were included to rule out DNA contamination. The elongation factor-1 (EF)
210 from *C. gigas* was chosen to normalize the viral gene expression. PCR efficiency (E) was
211 determined by drawing standard curves from a serial dilution analysis of the pool of cDNA to
212 ensure that E ranged from 99% to 108% for each primer pair.

213 The calculation of the relative mRNA levels was based on a comparative Ct method [31]. No
214 differences between Ct values were observed for EF among temperatures and between injection
215 types and times. Therefore, the relative quantification value of the samples was normalized with
216 EF and relative to the positive control, and was expressed as $2^{-\Delta\Delta Ct}$, $\Delta Ct = [Ct(cDNA\ sample) -$
217 $Ct(positive\ cDNA\ control)]$ and $\Delta\Delta Ct = \Delta Ct\ of\ cDNA - \Delta Ct\ of\ EF$.

218 Standard curves were performed for each primer pair using serial dilutions of total DNA, with
219 PCR efficacy ($E=10^{(-1/slope)}$) being subsequently calculated thanks to these curves [32].

220 Statistical analysis

221 Survival functions were computed according to [33]. Survival time was measured in hours
222 (experiments 1A and 2) or days (experiment 1C) from the onset of each experiment phase. The
223 data were read as the number of dead oysters within each tank at each count. Survival time curves

of recipients were compared using the Cox model (1972) after adjusting for temperature effect (21°C, 26°C, 29°C, experiments 1A, 1C and 2), acclimation (acclimated vs non acclimated, experiment 1A) or injection (OsHV-1 vs SSW, experiments 1C). In experiment 1A, the survival of control recipients was not included in the statistical model because it was 100 %. The proportionality of hazards (PH) was checked with Martingale residuals [34]. Because the PH assumption was violated, time dependent covariates representing the interaction of the original covariates and times were added to the model. Time (t) was defined as dichotomous: $t \leq 120$ h or $t > 120$ h. Custom hazard ratios were produced by means of contrasts.

Mixed-design ANOVAs were performed to assess differences in (i) OsHV-1 DNA in pathogen recipients (experiments 1A and 2), depending on temperature (3 levels, main plot), acclimation (2 levels, subplot) and time (4 levels, sub-subplot) and (ii) the viral gene expression in acclimated pathogen recipients (experiments 1A and 2), depending on temperature (3 levels, main plot) and time (2 levels subplot). The replication unit was the tank in which the temperature and infection treatments were applied. All mutual interactions among factors were tested, and Tukey's HSD was used as a *post hoc* test. The normality of residuals and homogeneity of variances were graphically checked, and the data were log(x+1) transformed where necessary. Statistical analyses were performed in SAS 9.4 software (SAS institute).

Results

Effect of high temperatures on OsHV-1 transmission, infection, and survival of oysters (experiment 1A)

The oysters injected with OsHV-1 suspension (pathogen donors) showed significant mortalities 48 hpi at the three tested temperatures (Fig. S1). Their final survival at 72 hpi was ranked as 29°C (72 ± 2.1%) >26°C (62 ± 2.1%) >21°C (46 ± 2.6%).

The survival of control recipients (oysters living in cohabitation with SSW injected oysters) was 100 ± 0 % irrespective of temperature and acclimation treatments (data not shown), but low levels of OsHV-1 DNA were occasionally detected in these animals ($<10^3$ cp mg^{-1} wet tissue, Table S1). Although control recipients were no longer considered SPF, absence of mortality suggest that they were healthy, and only the pathogen recipients were considered hereafter.

At the end of the cohabitation trial, the survival of pathogen recipients acclimated at 29°C was higher (85.7 ± 2.0%) than at 21°C and 26°C, where survival rates were 52.4 ± 3.1% and 43.9 ± 3.1% respectively (Fig. 2A). Survival of non-acclimated pathogen recipients was 5-9% lower than that of their acclimated counterparts irrespective of temperature. Mortality started 72 h post-cohabitation (hpc) at 26°C and 120 hpc at 21°C (Fig. 2A). Overall, mortality risk was lowest at 29°C (Table S2).

At the onset of the experiment, OsHV-1 DNA was not detected in SPF oysters. Between 0-24 hpc, the level of OsHV-1 DNA in pathogen recipients increased above 10^4 cp.mg^{-1} irrespective of temperatures, but rates of increase were the highest at 26°C and 29°C (Fig. 2B, Table S3). Between 24-48 hpc, the level of OsHV-1 DNA continued to increase at 21°C, remained high at 26°C, and initiated a decrease at 29°C. Therefore, at 48 hpc, the level of OsHV-1 DNA ranked as 26°C = 21°C > 29°C. Finally, between 48-96 hpc, OsHV-1 DNA decreased at 26°C and 29°C while it remained stable at 21°C. Therefore, at 96 hpc, OsHV-1 DNA ranked as 21°C > 26°C = 29°C. Interestingly, maximum values of OsHV-1 DNA were similar among temperatures

267 (1.9×10^6 cp.mg^{-1} at 21°C 48 hpc; 3.4×10^6 cp.mg^{1} at 26°C 24 hpc and at 29°C, 4.0×10^5 cp.mg^{-1} 24
268 hpc) but these maximum values were observed for longer at 26°C and 21°C than at 29°C.

269 The six viral Open Reading Frames (ORFs) selected in our study were expressed in pathogen
270 recipients at the three tested temperatures. Three of them were modulated by temperature (ORFs
271 27, 38 and 87). Gene expression levels were highest at 26°C, except ORF 87 at 48 hpc, which
272 exhibited the highest values at 21°C. At 29°C, viral gene expressions of these three ORFs were
273 lower than at 26°C and 21°C. Viral gene expression of the other ORFs (41, 67 and 99) increased
274 between 24 and 48 hpc (Fig. 2C and D).

275 *Effect of previous temperature treatments on recipient survival at 21°C (experiment 1B).*

276 The survival of both control and pathogen recipients placed at 21°C for 11 days was 100 ± 0 %
277 irrespective of previous temperature treatments (data not shown). Moreover, the new SPF oysters
278 placed in cohabitation with them showed no mortality.

279 *Effect of previous temperature treatments on oyster susceptibility to a second infection in a*
280 *farming area (experiment 1C).*

281 Both pathogen and controls recipients transferred to a farming area where OsHV-1 occurred
282 showed high mortalities. However, the survival of pathogen recipients was higher (75.9 ± 5.3%)
283 than that of control recipients (44.9 ± 9.9%, Fig. 3, Table S4). There was no effect of previous
284 temperature treatments on the survival of control recipients. In contrast, the survival of the
285 pathogen recipients infected at 29°C was lower (67.9 ± 10.8%) than those infected at 21°C and
286 26°C, where survival was 79.3 ± 3.7% and 80.6 ± 5.5% respectively. Throughout the whole
287 experiment 1, the survival of the pathogen recipients previously exposed to donors at 29°C

288 remained higher (56.6%) than at 21°C and 26°C, where survival was 41.9% and 32.0%
289 respectively.

290 *Effect of incubation temperature on the OsHV-1 suspension (experiment 2).*

291 As observed in experiment 1, the survival of control recipients was 100 ± 0 % but low levels of
292 OsHV-1 DNA were occasionally detected ($<10^2$ cp mg^{-1} wet tissue, Table S1). However, given
293 the absence of mortality and temperature effect on control recipients, only the pathogen recipients
294 were considered hereafter.

295 Pathogen recipients showed significant mortalities after 72 hpc (Fig. 4A, Table S5). At the end of
296 experiment 2, the survival of pathogen recipients ranked as a function of incubation temperature
297 of the viral suspension: 26°C (46 ± 3 %) < 21°C = 29°C (52 ± 2 %). The level of OsHV-1 DNA
298 and viral gene expression in pathogen recipients increased during the experiment but were not
299 influenced by the incubation temperature of the viral suspension (Fig. 4B and C).

300 **Discussion**

301 The major result of this study is that the survival of recipient oysters exposed to the virus at 29°C
302 (85.7% survival) was markedly higher than at 21°C and 26°C (52.4% and 43.9% respectively),
303 reflecting changes in host susceptibility and/or virus virulence. This concurs with a previous
304 study where recipients cohabited with field-infected donors [16]. These differences in survival
305 persisted when the pathogen recipients were all placed at 21°C, the optimal temperature for
306 OsHV-1 replication, and when they were re-exposed to OsHV-1 in the field. This result opens
307 new perspectives for mitigation measures using high temperature.

The differences in survival between recipients exposed to OsHV-1 at 21°C and 29°C coincided with differences in viral replication in the host. Although levels of OsHV-1 DNA increased from undetectable amounts to values higher than 10^4 DNA cp mg^{-1} (the threshold at which mortality generally occurs) for both temperatures during the first 24 hpc, it then decreased markedly in recipients at 29°C whereas it continued to increase at 21°C. A similar pattern was observed in SPF oysters having cohabited with field infected donors at these two temperatures [16]. In our study, the expression of three viral genes (ORF 27, 38 and 87) involved in regulation of a ring finger motif, DNA repairing and apoptosis inhibition [30] were lower in recipients at 29°C than at 21°C. High temperature may reduce the expression of some viral genes that could be essential to the development of the disease and viral cycle. Also, equally high expression levels of ORF 41, 67 and 99 at 21°C and 29°C suggest the induction of the OsHV-1 lytic cycle irrespective of temperature [35].

Survival of pathogen donors at 29°C was higher than at 21°C, as observed in pathogen recipients. This suggests that high temperature (29°C) decreased the susceptibility of oysters to OsHV-1. This, however, raises the possibility that viral shedding of pathogen donors at 29°C was lower than that at 21°C, which could have contributed to the increased survival of the pathogen recipients at 29°C.

We also found that these temperatures (21°C and 29°C) had no effect on the virus suspension *per se*. Indeed, the survival, level of OsHV-1 DNA and virus gene expression of recipients at 21°C having cohabited with donors injected with viral suspension incubated were all similar at 21°C or 29°C. Therefore, high temperature (29°C) had no effect on the infectivity (a measure of the ability of a disease agent to establish itself in the host) or the virulence (a measure of the severity of a disease) of OsHV-1.

Therefore, high temperature (29°C) decreased the susceptibility of oysters to OsHV-1 without altering virus infectivity and virulence; however, the underlying mechanism remains unknown. Temperature impacts all aspects of ectotherm physiology, including their immunity [36]. For instance, the antiviral response of oysters is stimulated at 22°C compared to 12°C [37]. High temperatures (32°C) induce overexpression of heat shock proteins 70 involved in the repression of white spot syndrome virus replication in shrimps [38].

At 26°C, the mortality of pathogen recipients started earlier than at 21°C and 29°C, survival was lowest, levels of OsHV-1 DNA were highest (between 12-48 hpc) and the expression of three viral genes was enhanced. Furthermore, the survival of pathogen recipients having cohabited with donors injected with viral suspension incubated at 26°C was lowest, but levels of OsHV-1 DNA and viral gene expression were similar among temperature treatments. Therefore, it appears that the virulence of OsHV-1 was enhanced at 26°C, although an increase in susceptibility of the host cannot be ruled out.

In our study, the survival of pathogen recipients at 26°C is the lowest as reported by [17]. However, in a previous study, survival of oysters exposed to OsHV-1 at 21°C was lower than at 26°C [16]. Discrepancies may reflect different methods of infection among studies. Here, the donors were injected with a viral suspension, whereas in [16] they were naturally infected by a short exposure to field conditions where OsHV-1 induced mortalities of oysters were occurring. Therefore, their study probably encompasses other microorganisms naturally encountered in the marine environment, including populations of pathogenic vibrios that influence oyster survival [22,39].

When temperature of pathogen recipients decreases from 29°C to 21°C, a temperature permissive for OsHV-1 replication, there was neither additional mortality nor disease transmission to the new SPF oysters. Although high temperature increases elimination of several viruses in oysters [40], herpesviruses are generally incurable and complete elimination from the host is unlikely [41]. Our results suggest that OsHV-1 did not reactivate at 21°C, or at least not sufficiently to induce mortality. However, OsHV-1 can persist in oysters for several months at low temperatures (<13°C) and can reactivate and kill the host after a thermal elevation to 21°C [42].

Recipients showed additional mortalities following the second exposure to OsHV-1 in the field. The survival of pathogen recipients previously infected at 29°C was lower than those infected at 21°C and 26°C, likely reflecting the persistence of a greater number of susceptible hosts at 29°C.

Throughout the whole experiment, the survival of recipients exposed to pathogen donors at 29°C was on average 20% higher than at 21°C and 26°C. Oysters first infected at 29°C seemed less susceptible to the virus than those first infected at 21°C and 26°C. For the second infection, the oysters were 1 ½ month older and therefore potentially less susceptible to the pathogen [43]. Also, the first exposure to OsHV-1 may have lowered the susceptibility of oysters to a second infection. A non-lethal exposure to a pathogen can enhance invertebrate immunity by immune priming [44,45]. Immune priming against OsHV-1 has already been reported for oysters injected with a synthetic viral analogue [46].

Oysters exposed to the virus at 29°C were less susceptible to OsHV-1 than at 21°C and 26°C over the long term, thus opening new perspectives for mitigation measures. Hyperthermia was already suggested as preventive method in shrimp farming to minimize the risk of a viral outbreak [47]. Regarding the potential of high temperature treatment to mitigate disease risk in

oyster farms, several questions need to be answered. What is the sanitary status of the oysters exposed to OsHV-1 at high temperature (OsHV-1-free or asymptomatic carrier)? What is the optimal treatment duration and temperature to optimize survival and minimize energy costs? How is it possible to practically implement a high temperature treatment on a production farm? Finally, what are the economic efficiency (i.e. the cost of disease control measures compared to ensuing benefits) and acceptability (the willingness of organizations to put into action disease control measures) of high temperature treatment?

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541

542

543 **Table 1.** List of all treatment combinations used in experiments 1A, 1B and 2 in the laboratory.
544 Abbreviations: SSW, synthetic seawater; A, acclimated; NA, non-acclimated.

Seawater temperature	Injection	Injection temperature	Replicate	Tank	Acclimation	Experiment

Seawater temperature	Injection	Injection temperature	Replicate	Tank	Acclimation	Experiment
21°C	SSW	21°C	1	1	A	1-2
			2	2	A	1-2
		26°C	1	3	A	2
			2	4	A	2
		29°C	1	5	A	2
			2	6	A	2
	OsHV-1	21°C	1	7	A-NA	1-2
			2	8	A-NA	1-2
			3	9	A-NA	1-2
		26°C	1	10	A	2
			2	11	A	2
			3	12	A	2
		29°C	1	13	A	2
			2	14	A	2
			3	15	A	2
26°C	SSW	21°C	1	16	A	1
			2	17	A	1
	OsHV-1	21°C	1	18	A-NA	1
			2	19	A-NA	1
			3	20	A-NA	1
29°C	SSW	21°C	1	21	A	1

Seawater temperature	Injection	Injection temperature	Replicate	Tank	Acclimation	Experiment
			2	22	A	1
	OsHV-1	21°C	1	23	A-NA	1
			2	24	A-NA	1
			3	25	A-NA	1

545

546

547 **Figure legends**

548 **Fig. 1.** Experimental design to investigate the effect of temperature on disease susceptibility of
549 the Pacific oyster *Crassostrea giga*s and virulence of the Ostreid herpesvirus type 1.
550 Abbreviations: OsHV-1, Ostreid herpesvirus type 1; SPF, specific-pathogen free oyster; SSW,
551 synthetic seawater.

552 **Fig. 2.** (A) Survival of oysters (pathogen recipients) having cohabited with donors injected with
553 OsHV-1 at 21°C, 26°C and 29°C with or without prior acclimation. (B) Quantification of OsHV-
554 1 DNA in pathogen recipients as a function of temperature. Data of acclimated and non-
555 acclimated oysters were averaged due to temperature × acclimation × time interaction not being
556 significant. Inset represents the significant interaction of temperature × acclimation. Data were
557 log (x+1) transformed. (C and D) Viral gene expression expressed as a delta threshold cycle
558 number (Ct) of OsHV-1 open reading frames (ORFs) relative to *C. gigas* elongation factor in live
559 acclimated pathogen recipients as a function of temperature. Inset represents the main effect of
560 time averaged for the six tested ORFs. The gene expression analyses were conducted only at 24
561 hpc and 48 hpc. For all analyses, values are means ± SE (n=3 tanks), and different letters indicate
562 significant differences. Only significant effects are represented.

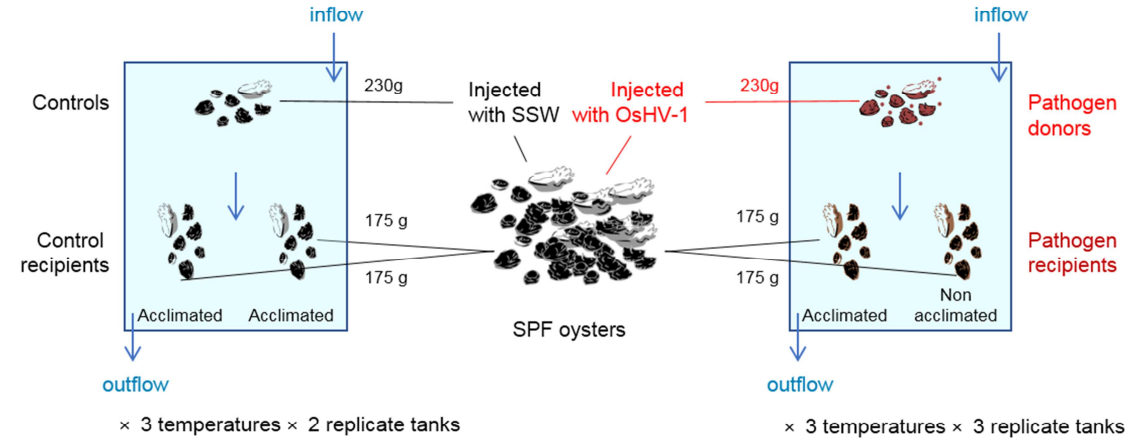
563 **Fig. 3**. Survival of pathogen recipient after being transferred to a farming area where OsHV-1
564 occurred as a function of temperature during the first exposure to OsHV-1 in the laboratory.

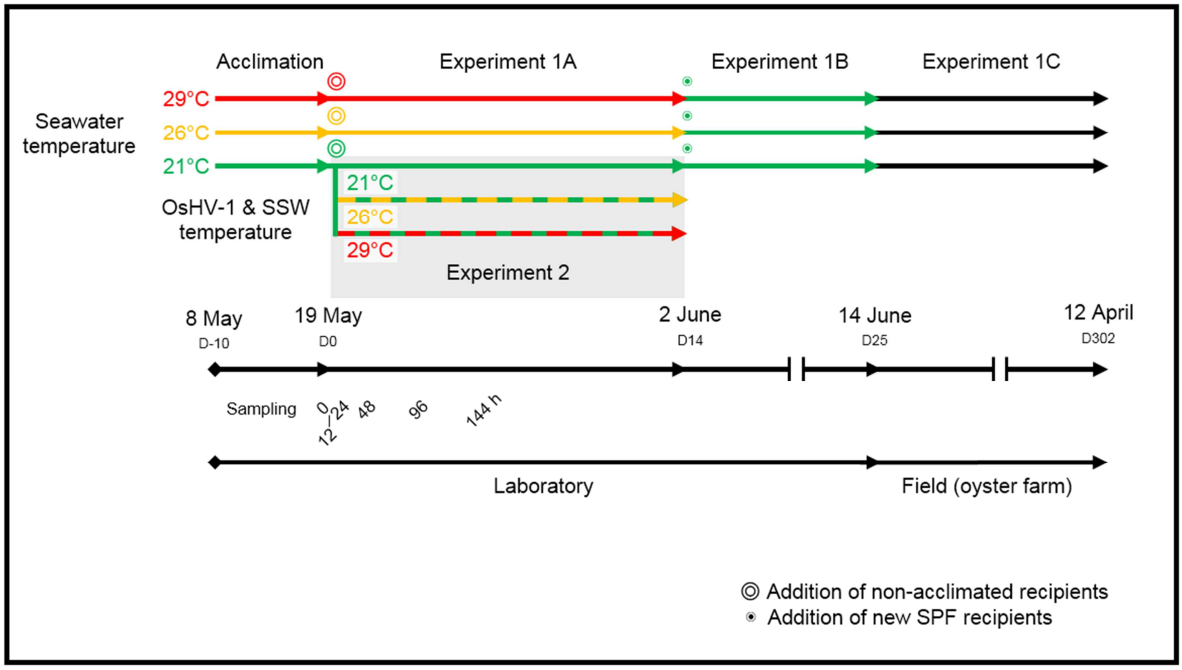
565 **Fig. 4.** (A) Survival of oysters (pathogen recipients) having cohabited with donors injected with
566 OsHV-1 incubated at 21°C, 26°C and 29°C for 5 hours. (B) Quantification of OsHV-1 DNA in
567 pathogen recipients as a function of incubation temperature of the viral suspension. Data of
568 recipients at 21°C, 26°C and 29°C were averaged because the interaction of temperature × time
569 was not significant (black line). Data were log (x+1) transformed. (C) Viral gene expression

570 expressed as a delta threshold cycle number (Ct) of OsHV-1 open reading frames (ORFs) relative
571 to *C. gigas* elongation factor 1 in live acclimated recipients as a function of time. The gene
572 expression analyses were conducted only at 24 hpc and 48 hpc. For all analyses, values are means
573 ± SE (n=3 tanks), and different letters indicate significant differences. Only significant effects are
574 represented.

575

Fig. 1.


inflow
Controls
230g
Injected with SSW
Injected with OsHV-1
230g
inflow
Pathogen donors
Control recipients
175 g
175 g
175 g
175 g
Pathogen recipients
Acclimated
Acclimated
SPF oysters
Acclimated
Non acclimated
outflow
outflow
× 3 temperatures × 2 replicate tanks
× 3 temperatures × 3 replicate tanks


Acclimation
Experiment 1A
Experiment 1B
Experiment 1C
Seawater temperature
29°C
26°C
21°C
OsHV-1 & SSW temperature
21°C
26°C
29°C
Experiment 2
8 May
D-10
19 May
D0
2 June
D14
14 June
D25
12 April
D302
Sampling
0
12
24
48
96
144 h
Laboratory
Field (oyster farm)
◎ Addition of non-acclimated recipients
◉ Addition of new SPF recipients

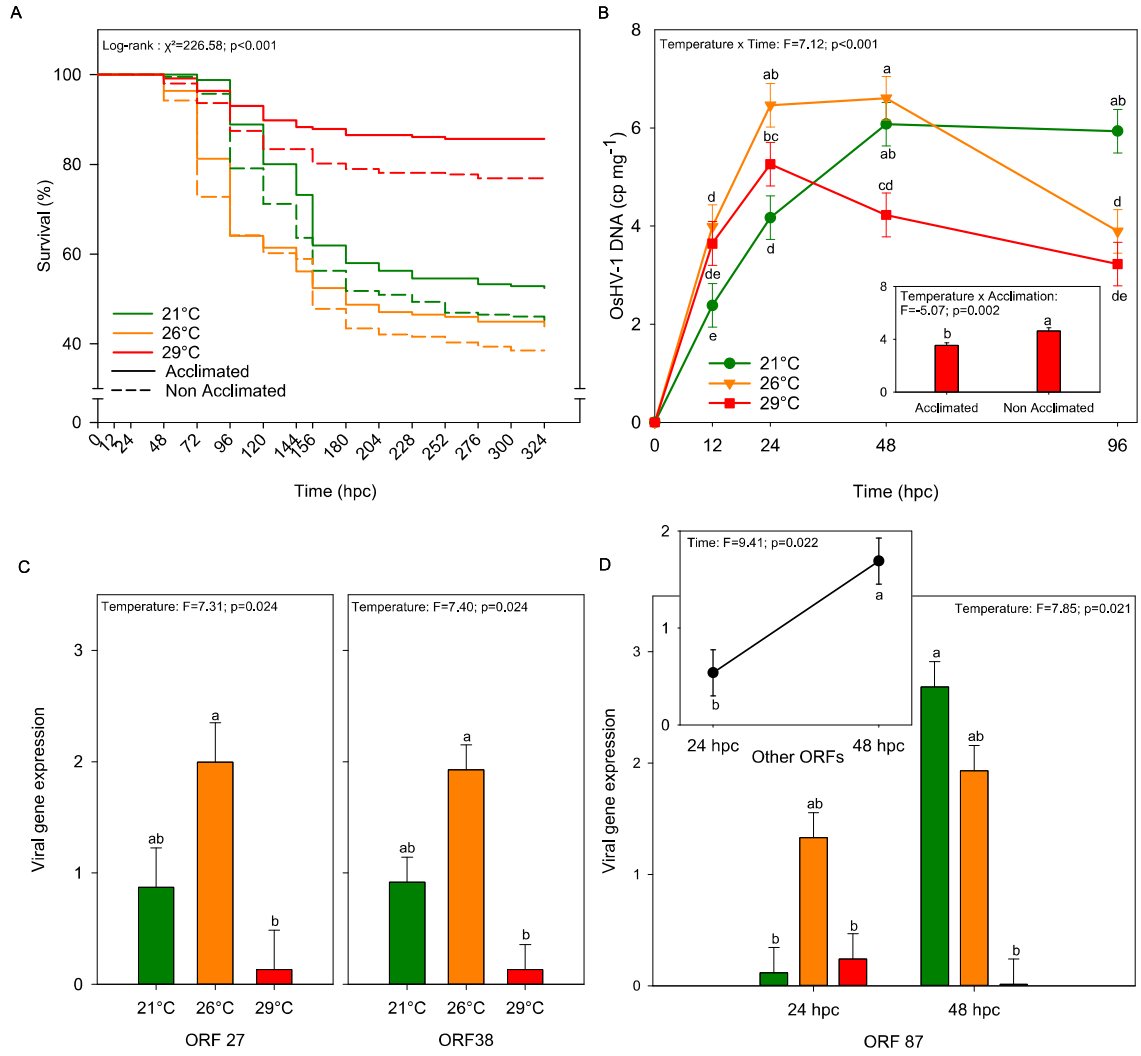

A
Log-rank : χ²=226.58; p<0.001
Survival (%)
Time (hpc)
21°C
26°C
29°C
Acclimated
Non Acclimated
B
Temperature x Time: F=7.12; p<0.001
OsHV-1 DNA (cp mg-1)
Time (hpc)
Temperature x Acclimation: F=-5.07; p=0.002
Acclimated
Non Acclimated
C
Temperature: F=7.31; p=0.024
Temperature: F=7.40; p=0.024
Viral gene expression
ORF 27
ORF38
D
Time: F=9.41; p=0.022
24 hpc
48 hpc
Other ORFs
Temperature: F=7.85; p=0.021
ORF 87

Fig. 3.

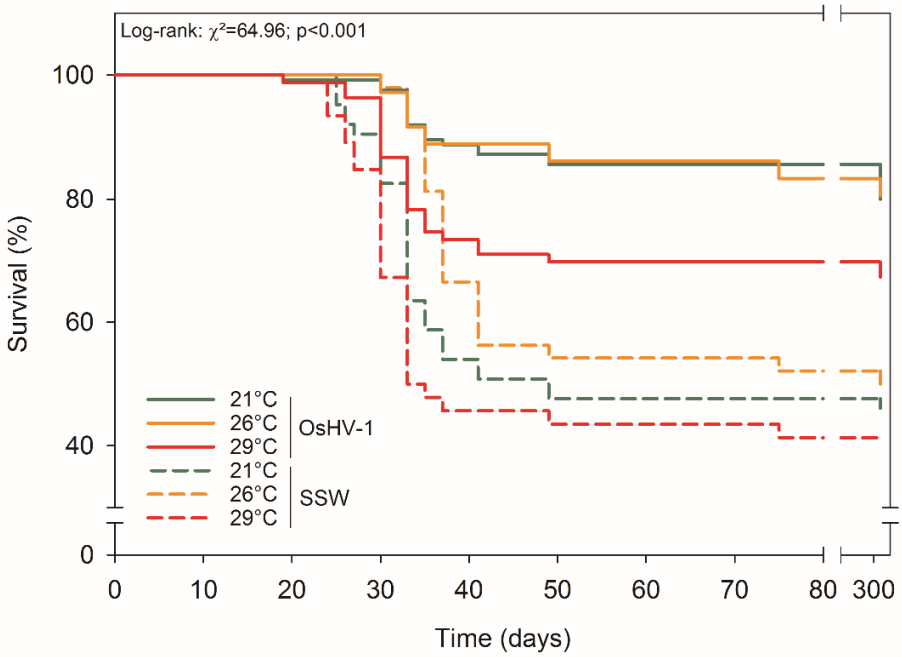
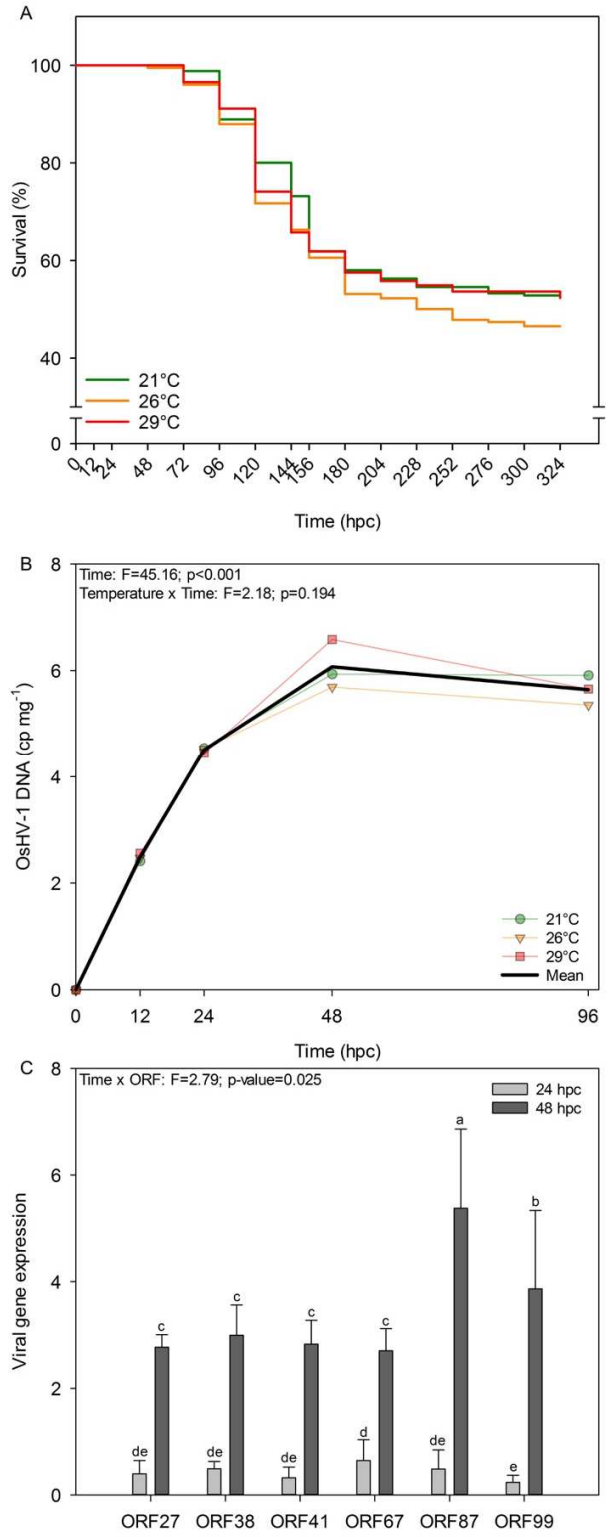


Fig. 4.


A
Survival (%)
100
80
60
40
0
21°C
26°C
29°C
0
12
24
48
72
96
120
144
156
180
204
228
252
276
300
324
Time (hpc)
B
Time: F=45.16; p<0.001
Temperature x Time: F=2.18; p=0.194
OsHV-1 DNA (cp mg-1)
8
6
4
2
0
21°C
26°C
29°C
Mean
0
12
24
48
96
Time (hpc)
C
Time x ORF: F=2.79; p-value=0.025
24 hpc
48 hpc
Viral gene expression
8
6
4
2
0
de
c
de
c
de
c
d
c
de
a
e
b
ORF27
ORF38
ORF41
ORF67
ORF87
ORF99