Aquaculture 408-409 (2013) 51–57

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

# Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles?☆

Dégremont Lionel *, Tanguy Guyader [1], Delphine Tourbiez, Jean-François Pépin

*Ifremer, SG2M, LGP2M, avenue Mus de Loup, 17390 La Tremblade, France*

ARTICLE INFO

*Article history:*
Received 12 April 2013
Received in revised form 22 May 2013
Accepted 24 May 2013
Available online 4 June 2013

*Keywords:*
Mortality
*Crassostrea gigas*
Disease resistance
Ostreid herpesvirus OsHV-1
Horizontal transmission
Pacific oyster

ABSTRACT

Massive mortality outbreaks, mostly affecting spat *Crassostrea gigas*, have been reported in France since 2008. Disease investigations revealed that most of the mortality events are related to the detection of the Ostreid herpesvirus 1 (OsHV-1 µVar). Meanwhile, selection to improve the survival in juvenile *C. gigas* has been successfully implemented in this context and selected oysters were found disease-resistant to OsHV-1 infection. This paper reports the first investigation of the horizontal transmission of the disease throughout cohabitation trials within batch and between batches using unselected and selected oysters for their higher survival. Two batches, unselected (AC) and selected (AR), were produced in February 2009 and deployed in the field in August 2009. Mortality associated with OsHV-1 detection occurred within the first two weeks of the deployment to reach 19% and 56% for the AR and AC batches, respectively. The remaining alive oysters were then brought into the laboratory in March 2010 for the cohabitation trials to assess the potential horizontal transmission of OsHV-1 within batch or between one of the batches and a third. The third batch (J) was produced using unselected oysters in August 2009 and it was always kept in the hatchery and the nursery to isolate it from the mortality risk factors. At the end of the cohabitation trials in July 2010, no mortality was found for the cohabitation trials between AR and J oysters as well as for the AR oysters and also for the J oysters grown alone in a tank. Conversely, high mortality was reported for the cohabitation trials using the J and AC batches as well as for the AC oysters alone. For the later, mortality occurred 3 days post-spawning while the AR oysters, which also spawned the same day, did not suffer of any abnormal mortality. Disease screening revealed that OsHV-1 was not detected for the J batch in March 2010, while 4 and 46% of the AR and AC oysters were found positive seven months post-mortality, indicating a difference in potential reservoir role within the *C. gigas* species. Our study clearly revealed the horizontal transmission of the disease from unselected asymptomatic adult to juvenile *C. gigas*, inducing mortality, and that OsHV-1 resistant oysters may offer one way to limit the spread of the disease and the potential reservoir role. Mortality due to OsHV-1 and its kinetic depended of the seawater temperature, which appeared as a strong risk factor that can reactivate the virus from latent-like or sub-clinical infection in asymptomatic adults. The lower threshold of 14 °C to observe mortality due to OsHV-1 was evidenced even if its replication was activated during the two previous weeks when seawater temperature was 16 °C. Finally, a sub-clinical level of virus was observed in AC asymptomatic oysters, it can be reactivated and cause viral replication, suggesting latency-like infection can exist in OsHV-1.



## 1. Introduction

Since 2008, massive mortality outbreaks routinely occur in *Crassostrea gigas* in France mostly affecting the juvenile oysters. Disease investigations highlighted the strong implication of the Ostreid herpesvirus 1 (OsHV-1) as the main cause of the mortality in juvenile oysters in field conditions (Dégremont, 2011; Segarra et al., 2010), confirming its role in previous mortality events reported between 1992 and 2007 (Dégremont et al., 2010a; Garcia et al., 2011; Renault et al., 1994). Even if a newly described genotype of OsHV-1 named µVar was reported since 2008 (Segarra et al., 2010), it is not possible to figure out if this genotype is more virulent than other genotypes described previously. Meanwhile, breeding programs for improving the survival in juvenile *C. gigas* during the summer mortality phenomenon showed a strong genetic basis (Dégremont et al., 2007, 2010b). The resistant oysters, thereby named R, selected from 2001 to 2003, for which OsHV-1 was detected in several mortality events in nursery and under laboratory conditions (Dégremont, 2003; Dégremont et al., 2010a), were also resistant to the new genotype (Dégremont, 2011).



* Corresponding author. Tel.: +33 5 46 76 26 30; fax: +33 5 46 76 26 11.
*E-mail address:* lionel.degremont@ifremer.fr (D. Lionel).
[1] Current address: France Naissain, Polder des champs, 85230 Bouin, France.

0044-8486/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2013.05.025

Dégremont (2011) also showed that R oysters were all infected but oysters were capable to limit the infection compared to unselected oysters as well as either to eliminate the virus of their tissue or decrease the quantity of viral DNA below the threshold of the real-time PCR technique after the peak of mortality. Together, these data open new investigations regarding the horizontal transmission of OsHV-1 between various stages of *C. gigas* using selected or unselected stocks.

To date, only a few studies have investigated the horizontal transmission of OsHV-1 between *C. gigas* stocks. The first report of a successful experimental transmission of OsHV-1 was reported among larvae (Le Deuff et al., 1994). A similar approach had been successful between infected juvenile oysters to naïve larvae, which indicates the spread of OsHV-1 between infected hosts in the field is possible (Burge and Friedman, 2012). Cohabitation trials between healthy and experimentally infected oysters also showed the horizontal transmission of the virus (Schikorski et al., 2011a). However, all these approaches used unselected oyster stocks.

In our study, selected and unselected oyster batches were produced and then tested in the field where mortality mainly due to OsHV-1 occurs at favorable seawater temperatures (Garcia et al., 2011). At one year old, surviving oysters were then transferred into the laboratory to test transmission of the virus to naïve juvenile oysters. The use of juvenile oysters instead of adults was based on higher susceptibility of juveniles than adults in mortality events (Dégremont et al., 2010c) which also corresponds to higher susceptibility of juveniles to OsHV-1 compared to adults (Arzul et al., 2002; Renault and Novoa, 2004). The purpose of this study is to analyze the effect of selection for higher resistance to OsHV-1 to the horizontal transmission of the disease.

## 2. Materials and methods

### 2.1. Oyster batches

Three batches were produced at the IFREMER hatchery in La Tremblade. The first two batches were spawned on February 23rd 2009, producing the selected oysters for higher resistance to mortality, and so to OsHV-1 infection, and the unselected oysters. Details are given in Dégremont (2011) and to summarize, selected oysters are the sixth generation of one R family developed during the MOREST program while unselected oysters used wild animals sampled in the Marennes-Oléron Bay. Each cross used 30 parents and 20 parents for the selection, hereafter AR, and control, hereafter AC, batches respectively. Oysters were grown at the IFREMER hatchery in La Tremblade and then at the IFREMER nursery in Bouin until their field deployment on August 2009. This method allows producing oyster batches without any abnormal mortality, in particular those caused by OsHV-1, due to UV filtration used at the hatchery, known to be efficient at inactivating the virus (Schikorski et al., 2011b), and the seawater flow supplement at the nursery.

The third batch, hereafter the J batch, was spawned on August 2009, using the same wild population brood stock and the same husbandry protocols. In March 2010, 2400 J oysters were transferred into the laboratory facilities for the horizontal transmission trials and 24 oysters were sampled for initial disease diagnosis.

### 2.2. Field study

The AR and AC batches were deployed at Agnas in the Marennes-Oléron Bay on August 6th 2009. For each batch, two bags of 5 kg were attached to a rack, which represented 1440 and 1490 oysters for the AR and AC batches, respectively. Dead and alive oysters were counted on August 20th, in October 2009 and in March 2010. On March 4th 2010, 400 oysters per batch were transferred into the laboratory for the horizontal transmission trials. Disease diagnoses were performed on August 20th from 6 live AR oysters and 6 moribund oysters from both batches, as well as 24 oysters per batch sampled on March 4th 2010.

### 2.3. Horizontal transmission trials

In March 2010, the AR, AC and J batches were brought to the IFREMER hatchery in La Tremblade for the horizontal transmission trials between individuals within batch and between adult and juvenile oysters. For adults, the oysters of the AR and AC batches were selected according to their size in order to have the same biomass in each tank. The mean individual weight was then 14 g. For the juveniles, mean individual weight was 0.3 g for the J batch.

Two sets of 5 tanks were settled corresponding to two seawater conditions, one with flow-through UV-filtered seawater heated at 20–22 °C, and the second one with flow-through UV-filtered unheated seawater. Indeed, seawater temperature is a key parameter to observe mortality due to OsHV-1 in the field, and recent data showed the threshold beyond which mortality could occur around 16 °C (Pernet et al., 2012). Complete water replacement took place over each hour, and water temperatures were continuously monitored every 10 min using ThermoTrack probes (Progesplus, 59780, Willems, France). For each set, three tanks were dedicated to hold one batch alone corresponding to the "cohabitation trial" among individuals within batch: one tank containing 100 AR oysters, another one containing 100 AC oysters, and the last one containing 400 J oysters. The two other tanks were dedicated for the cohabitation trials between one adult batch, either selected or not, and the J batch: 100 AR oysters with 400 J oysters or 100 AC oysters and 400 J oysters. Mortality was recorded three times per week from March 2010 to July 2010 and daily during mortality events. Total weight of the live oysters was recorded at the end of the experiment and any event, like natural spawn, recorded. Finally, three disease samplings were conducted in April, May and June 2010. For each sampling, 12 live oysters per batch in each tank were randomly sampled. Additionally, all dead oysters with tissue were frozen and disease diagnoses were performed for 2 to 6 animals per batch for each relevant mortality event.

### 2.4. Disease diagnosis

All alive and moribund oysters sampled were individually tested for OsHV-1. Additionally, all moribund oysters were also individually screened for *Vibrio aestuarianus*. As regards *Vibrio splendidus*, it was not tested as it is a dominant *Vibrio* species in seawater (Le Roux et al., 2009), which is considered as an opportunistic pathogen (Gay et al., 2004), and there were not routine tool to discriminate virulent from non virulent strains of *V. splendidus*.

DNA detection and quantification of pathogens was carried out using highly sensitive real-time quantitative PCR tests (qPCR) as described in Pépin et al. (2008) and Saulnier et al. (2009) for OsHV-1 and *V. aestuarianus* respectively. Briefly, around 50 mg of fresh oyster soft tissue (mantle and gills) was crushed for total DNA extraction using the Qiagen QIAamp® tissue mini kit according to the manufacturer's protocol (QIagen). Final elution of the DNA was performed with 100 µl of double-distilled water, and DNA concentration was performed spectrophotometrically (NANODROP®). Dilution of DNA samples was done in order to have a final DNA concentration of 4 ng/µl. Each DNA sample was tested in duplicate qPCR tests using 20 ng of DNA in a final reaction volume of 25 µl.

The OsHV-1 DNA detection and quantification was carried out using SYBR® Green real-time PCR protocol described by Pepin et al. (2008), adapted to use primers targeting OsHV-1 DNA polymerase sequence (ORF 100) denoted OsHVDPFor: 5′ATTGATGATGTGGATAATC TGTG3′ and OsHVDPRev: 5′GGTAAATACCATTGGTCTTGTTCC3′ (Webb et al., 2007). Absolute quantification was based on standard curve prepared using dilutions of viral DNA suspension and included for each PCR

test plate. Results were expressed as viral DNA copy number per mg of oyster tissue.

Similarly, the detection and quantification of *V. aestuarianus* DNA was carried out using a previously published real-time PCR protocol based on a Taqman® chemistry, targeting the dnaJ gene (Saulnier et al., 2009). The primers used were DNAj F: 5′GTATGAAATTTTAA CTGACCCACAA3′ and DNAj R: 5′CAATTTCTTTCGAACAACCAC3′ associated to the DNAj probe: Texas Red-TGGTAGCGCAGACTTCGGCGAC-BHQ2. The method was adapted for direct detection of bacterial DNA from the whole DNA extracted from oyster tissue.

### 2.5. Statistical analyses

Mortality and OsHV-1 prevalence (frequency detection) were analyzed using the GENMOD procedure SAS® software version 9 with a logit transformation and a binomial distribution. Various models were used depending on effects hypothesized. As no *V. aestuarianus* was found in any oyster in this study, data on this pathogen was not included in any of the statistical analyses performed.

The logistic regression model used for the mortality and OsHV-1 prevalence in March 2010 was:

$$\text{Logit}(Yi) = \log(Yi/(1-Yi)) = \mu + \text{batch } i$$

where Y*i* is the probability of an unfavorable response (dead for the mortality; presence of the OsHV-1) for the *i*th batch (AR, AC and J) and $\mu$ is the intercept. Multiple comparison tests were conducted using the least squares means statement and the PDIFF option (Littell et al., 2002).

The binomial models used for the analysis of variance of the mortality on July 15th for the within batch or between batches cohabitation trials were respectively:

$$\text{Logit}(Yij) = \log(Yij/(1-Yij)) = \mu + \text{batch } i + \text{seawater condition j} + \text{batch } i \times \text{seawater condition j}$$

$$\text{Logit}(Yij) = \log(Yij/(1-Yij)) = \mu + \text{tank } i + \text{seawater condition j} + \text{tank } i \times \text{seawater condition j}$$

where Y*i* is the probability of an unfavorable response (dead for the mortality) for the *i*th batch (AR, AC, J) or the *ith* tank (tanks 1: AC and J versus tank 2: AR and J) in the *jth* seawater condition (heated and unheated).

Finally, similar statistic approaches as described above was used to analyze the prevalence of OsHV-1 on June 16th but by adding a covariate in the models, which was the logit transformation of the prevalence on March 4th.

All tests were considered significant at $P < 0.01$.

## 3. Results

### 3.1. Field mortality

A peak of mortality was observed within two weeks after deployment to reach 16 and 55% for the AR and AC batches respectively on August 20th 2009 (Fig. 1). Any further mortality was observed from September 2009 to March 2010. Cumulative mortality reached 19 and 56% for the AR and AC batches respectively and was statistically significant.

### 3.2. Seawater temperature during the horizontal transmission trials in laboratory

Water temperature recorded for the two seawater conditions are shown in Fig. 2. For the heated seawater condition, temperature was 21.5 ± 1.3 °C, while in unheated conditions, seawater temperature raised from 4 °C in March to 25 °C in July. The threshold temperature of 16 °C, beyond which mortality due to OsHV-1 could be observed in the field, was reached for the first time in April 22nd in our laboratory trial. Seawater temperature then ranged from 16 to 17 °C for 12 days before it cooled down to 13 °C over a period of 2 days. Then seawater temperature regularly increased to stay above 16 °C from May 11th.

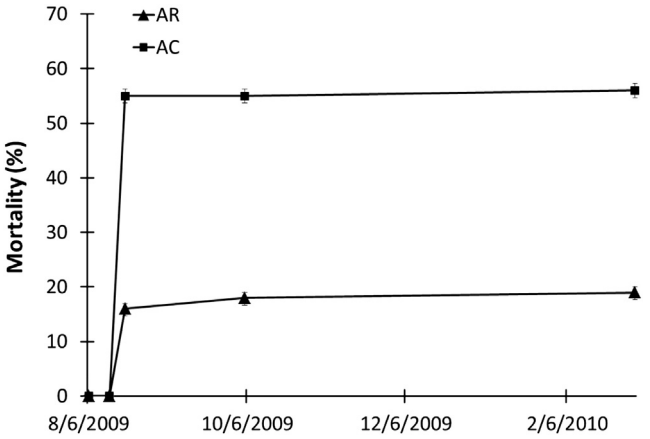


**Fig. 1.** Cumulative mortality for the AR and AC batches from field (bars represent the standard deviation of the mortality rate). AR and AC are selected oysters, 'resistant' to summer mortality and OsHV-1 infection, and unselected oysters respectively.

### 3.3. Mortality

For the cohabitation trials within batch, cumulative mortality of the AR and J batches remained low (<5%) throughout the experiment in both seawater conditions (Table 1). Similar finding was observed for the cohabitation trials between the AR and J batches.

As regards all other conditions involving the AC batch, significant higher cumulative mortality was recorded and ranged from 21 to 55% for the AC batch and ranged from 50 to 66% for the J batch (Table 1). However, the onset of the mortality did not occur simultaneously between the cohabitation trials and between batches. For the cohabitation trial within batch, only one mortality event was observed and it started at the same period between June 4th and June 7th for the AC batch (Fig. 3(A), (B)). In the cohabitation trials between batches, both AC and J batches experienced high and rapid mortality on May 17th and 18th for the heated condition and in much lesser extent for the J batch on June 9th (Fig. 4(A)). Conversely, mortality first started for the J batch for the unheated conditions from May 7th and it was much slower than in heated conditions, as it lasted 10 days (Fig. 4(B)). As in heated condition, a second mortality

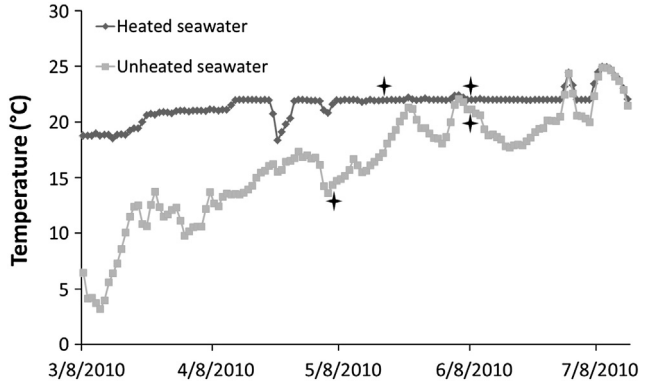


**Fig. 2.** Kinetics of seawater temperature during the horizontal transmission trials at laboratory from March 2010 to July 2010. Stars below and above the lines indicate mortality events in one of the batches tested in unheated and heated seawater condition respectively.

**Table 1**
Cumulative mortality and standard deviation (%) at the end of the horizontal transmission of OsHV-1 trials within or between batches in July 2010.

| Cohabitation | Batch | Heated seawater | Unheated seawater |
|---|---|---|---|
| Within batch | AR | 3.0 ± 1.7 | 4.6 ± 2.1 |
| | AC | 48.3 ± 5.0 | 40.1 ± 4.9 |
| | J | 0.3 ± 0.2 | 0.3 ± 0.5 |
| Between batches | AR | 3.2 ± 1.8 | 2.1 ± 0.7 |
| | J | 0.3 ± 0.3 | 0.0 ± 0.0 |
| | AC | 54.8 ± 5.0 | 21.3 ± 2.0 |
| | J | 66.1 ± 2.4 | 49.4 ± 5.0 |

event was observed for the J batch on June 9th. Finally, AC oysters experienced chronic mortality throughout the cohabitation trials to reach 21% on July 15th (Fig. 4(B)).

### 3.4. Disease diagnosis

#### 3.4.1. V. aestuarianus detection and quantification

It is noteworthy that no *V. aestuarianus DNA* was detected in any batch at any time, in alive or dead animals (data not shown).

#### 3.4.2. OsHV-1 detection and quantification

*3.4.2.1. Field condition.* The herpes virus OsHV-1 was detected in all animals tested except in one alive specimen. Mean viral load was high for the moribund oysters ($>10^7$ DNA copies of OsHV-1 per mg of fresh oyster tissue) and low to moderate for the alive oysters ($<10^4$).

*3.4.2.2. Start of the cohabitation trials on March 4th 2010.* At the beginning of the cohabitation trials, without any mortality, OsHV-1 was detected in 4 and 46% for the AR and AC oysters respectively, while it was not detected in any of the 24 J oysters diagnosed (Table 2). The AC batch had significant higher prevalence for OsHV-1 than the two other batches. The mean viral load of the animals found positive to OsHV-1 was weak, lesser than $10^2$ for the AR oysters and 1.2 $10^3$ for the AC oysters (Table 3).

*3.4.2.3. During the cohabitation trials.* Prevalence of OsHV-1 and mean viral load for the alive animals testing positive, remained very low for the AR batch in both seawater conditions and both cohabitation trials (Tables 2 and 3). Similarly, OsHV-1 was not detected in any of the 144 J oysters screened throughout the duration of the cohabitation trials within batch and between batches when adults were the AR oysters.

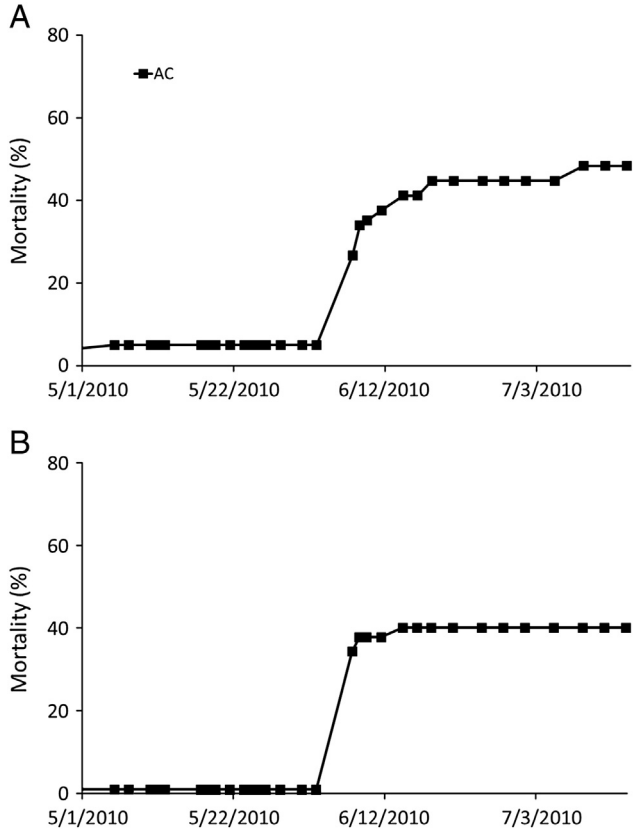


**Fig. 3.** Cumulative mortality for the AC batch in heated (A) and unheated (B) seawater conditions for the cohabitation trials within batch.

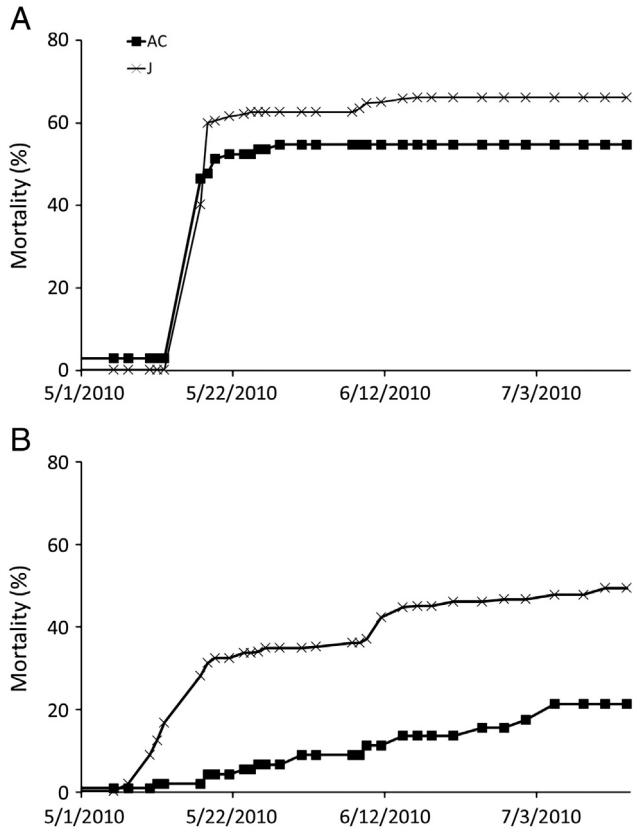


**Fig. 4.** Cumulative mortality for the AC and J batches in heated (A) and unheated (B) seawater conditions for the cohabitation trials between batches.

**Table 2**
Prevalence (%) of OsHV-1 for alive animals in unheated and heated (in italic) seawater conditions from March to June 2010.

| Cohabitation condition | Seawater condition | Batch | March 4th | April 12th | May 11th | June 16th |
|---|---|---|---|---|---|---|
| Within batch | Unheated | AR | 4 | 8 | 0 | 0 |
| | *Heated* | *AR* | *4* | *8* | *8* | *0* |
| | Unheated | AC | 46 | 33 | 16 | 66 |
| | *Heated* | *AC* | *46* | *66* | *33* | *91* |
| | Unheated | J | 0 | 0 | 0 | 0 |
| | *Heated* | *J* | *0* | *0* | *0* | *0* |
| Between batches | Unheated | AR | 4 | 4 | 0 | 8 |
| | | J | 0 | 0 | 0 | 0 |
| | *Heated* | *AR* | *4* | *0* | *16* | *8* |
| | | *J* | *0* | *0* | *0* | *0* |
| | Unheated | AC | 46 | 50 | 66 | 66 |
| | | J | 0 | 0 | 58 | 41 |
| | *Heated* | *AC* | *46* | *75* | *33* | *66* |
| | | *J* | *0* | *0* | *0* | *41* |

**Table 3**
Mean viral load of OsHV-1 (DNA copies of OsHV-1 per mg of fresh oyster tissue) in alive animals detected positive in unheated and heated (in italic) seawater conditions from March to June 2010.

| Cohabitation condition | Seawater condition | Batch | March 4th | April 12th | May 11th | June 16th |
|---|---|---|---|---|---|---|
| Within batch | Unheated | AR | $<10^2$ | $<10^2$ | ND[a] | ND[a] |
| | *Heated* | *AR* | *$<10^2$* | *$<10^2$* | *$<10^2$* | *ND[a]* |
| | Unheated | AC | $1.2 \cdot 10^3$ | $3.2 \cdot 10^3$ | $1.6 \cdot 10^3$ | $3.0 \cdot 10^4$ |
| | *Heated* | *AC* | *$1.2 \cdot 10^3$* | *$4.6 \cdot 10^3$* | *$9.0 \cdot 10^4$* | *$5.0 \cdot 10^4$* |
| | Unheated | J | ND[a] | ND[a] | ND[a] | ND[a] |
| | *Heated* | *J* | *ND[a]* | *ND[a]* | *ND[a]* | *ND[a]* |
| Between batches | Unheated | AR | $<10^2$ | $8.2 \cdot 10^2$ | ND[a] | $<10^2$ |
| | | J | ND[a] | ND[a] | ND[a] | ND[a] |
| | *Heated* | *AR* | *$<10^2$* | *ND[a]* | *$<10^2$* | *$<10^2$* |
| | | *J* | *ND[a]* | *ND[a]* | *ND[a]* | *ND[a]* |
| | Unheated | AC | $1.2 \cdot 10^3$ | $2.0 \cdot 10^3$ | $9.3 \cdot 10^3$ | $1.1 \cdot 10^5$ |
| | | J | ND[a] | ND[a] | $3.2\ 10^7$ | $4.9\ 10^4$ |
| | *Heated* | *AC* | *$1.2 \cdot 10^3$* | *$4.0 \cdot 10^3$* | *$3.2 \cdot 10^4$* | *$6.3 \cdot 10^3$* |
| | | *J* | *ND[a]* | *ND[a]* | *ND[a]* | *$4.6 \cdot 10^3$* |

[a] ND: no OsHV-1 detection in all oysters screened.

Regarding the AC batch, prevalence of OsHV-1 ranged from 16 to 91% and the mean viral load of the oysters detected positive was moderate from $10^3$ to $10^5$ DNA copies per mg of fresh tissue (Tables 2 and 3). For the within batch cohabitation trials, the AC oysters had significant higher prevalence than the AR and J batches on June 16th 2010, and reached 66 and 91% for the unheated and heated seawater conditions respectively (Table 2). For the cohabitation trials between batches, the mean prevalence of OsHV-1 on June 16th was 66% for the AC oysters and 41% for the J oysters, which was significantly higher than tanks containing together the AR (8%) and J (0%) oysters (Table 2). For the first time, OsHV-1 was detected in the J oysters when those are grown along with the AC oysters. The first positive detection of OsHV-1 among the alive J oysters was found on May 11th in the unheated seawater condition, while it was on June 16th in the heated seawater condition (Table 2). Similarly, mean viral load was high for the animals screened in May ($>10^7$), while it was moderate in June ($10^3$–$10^4$) (Table 3).

During mortality events, according to the tanks and conditions, moribund or dead animals were also sampled and tested for OsHV-1 detection (Table 4). Except two individuals AC from unheated seawater condition, all samples were positive for OsHV-1 DNA detection (100%) with very high viral loads ($>10^8$).

### 3.5. Growth and spawn

It is important to report here that natural spawns occurred the same day on June 4th for the AR and AC batches in the within batch cohabitation trials and in both seawater conditions. At the end of the experiment on July 15th, mean individual weight was 26, 24 and 7 g for the AR, AC and J batches respectively for the within cohabitation trials, while it was 23, 24 and 4 g for the between batches cohabitation trials.

## 4. Discussion

The three major findings of this study, beside that all significant mortality events were related to OsHV-1, are clearly (1) the absence of horizontal transmission of OsHV-1 from the selected oysters, although some of AR oysters were infected, to the unselected juvenile oysters, (2) the observance of a mortality event related to OsHV-1 in the unselected juveniles from infected asymptotic unselected adults and (3) the evidence that OsHV-1 µVar latent-like infection or sub-clinical infection can occur in asymptomatic oysters surviving to primary infection, which can transmit the disease after reactivation of the virus.

Ultimately, no detectable *V. aestuarianus* DNA were evidenced in samples tested during field or cohabitation trials. All moribund oysters displayed high viral loads (Table 4) corresponding to lytic infection associated with very high-level replication, when virus particles burst or lyse host cells. Additionally, OsHV-1 is considered as the main cause of mortality in *C. gigas* when viral load exceeds $10^5$ DNA copies per mg (Oden et al., 2011; Pepin et al., 2008; Sauvage et al., 2009), suggesting that mortalities occurring during this study can be attributed to this pathogen alone.

It was clear that AC and AR batches were infected by OsHV-1 within the first two weeks after their field deployment and experienced mortality due to the disease as showed by Dégremont (2011). The difference in mortality between the two stocks should reflect that AR oysters are capable to limit the infection and so, exhibit lower mortality than unselected oysters as well as lower prevalence of OsHV-1 after the peak of mortality as demonstrated by Dégremont (2011).

It is the first time that prevalence of OsHV-1 was checked 7 months post-mortality. Disease diagnosed revealed a much lower

**Table 4**
Prevalence (%) and viral load (DNA copies of OsHV-1 per mg of fresh oyster tissue) of OsHV-1 for moribund animals sampled during mortality event.

| Cohabitation condition[a] | Seawater condition | Date | Batch | Number analyzed | Prevalence of OsHV-1 | Mean viral load |
|---|---|---|---|---|---|---|
| Within batch | Unheated | 06/07/10 | AC | 6 | 100 | $8.0 \cdot 10^8$ |
| | *Heated* | *06/07/10* | *AC* | *6* | *100* | *$1.9 \cdot 10^9$* |
| Between batches | Unheated | 05/07/10 | J | 7 | 100 | $7.3 \cdot 10^8$ |
| | | 05/18/10 | AC[b] | 2 | 0 | 0 |
| | *Heated* | *05/17/10* | *J* | *6* | *100* | *$4.1 \cdot 10^8$* |
| | | *05/17/10* | *AC* | *6* | *100* | *$3.1 \cdot 10^9$* |
| | Unheated | 06/09/10 | J | 3 | 100 | $3.7 \cdot 10^{10}$ |
| | | 06/09/10 | AC | 2 | 100 | $3.4 \cdot 10^{10}$ |
| | *Heated* | *06/09/10* | *J* | *5* | *100* | *$2.3 \cdot 10^{10}$* |
| | | *06/09/10* | *AC* | NA[c] | | |

[a] Between batches means between batches AC and J.
[b] No AC moribund oysters for the 40 previous days.
[c] NA: not available because any AC oysters died from May 28th to the end of the trials.

prevalence of OsHV-1 for the AR oysters than for the AC oysters indicating a difference in potential reservoir role within the *C. gigas* species and confirming a previous study showing that OsHV-1 can be detected in asymptotic adults (Arzul et al., 2002). The low viral load for positive adults in March was very close to those observed by Sauvage et al. (2009) in surviving *C. gigas* juveniles three months after a disease outbreak. These DNA detections several months post-mortality should not be due to a mechanical carriage of the virus by oysters through contaminated seawater in their mantle cavity. Thus, a part of the surviving population keeps a sub-clinical infection with low level of virus in their tissues and become asymptomatic oysters, suggesting latency rather than productive infection. Currently, no test is available to discriminate if the virus is latent or sub-clinical, and further research is needed to investigate a specific Latency Protein antibody or RT-qPCR targeting specific viral RNA (Jaber, 2009).

Based on the husbandry practices in our hatchery and nursery, on repeated negative results in all qPCR tests throughout the duration of the cohabitation trials, and the lack of abnormal mortality, we assume that J oysters were not infected and stayed free of OsHV-1 infection excepted when those were grown along with the AC oysters (Tables 1 and 2). Our results indicate that UV-filtration system was efficient to prevent the disease in the flow-through seawater, as demonstrated for Dermo and MSX infection in *Crassostrea virginica* (Ford et al., 2001). Interestingly, horizontal transmission of OsHV-1 from the AR oysters to the J oysters was not possible in our study, even if some AR oysters were infected. Thus, selected oysters had several advantages over unselected oysters with lower mortality, lower role of reservoir and limitation of spreading the disease as it is not possible to affirm that horizontal transmission of the disease from AR oysters to unselected oysters cannot occur.

Horizontal transmission of OsHV-1 was evidenced among individuals for the AC batch but also from AC oysters to J oysters. Meanwhile, several interesting results emerged from each seawater condition or cohabitation trial. The first concerned the within cohabitation trials for the AC oysters. It is remarkable to observe that mortality related to OsHV-1 was observed 3 days post-spawning for the AC oysters and not for the AR oysters. Obviously, AC oysters have higher susceptibility to stress than AR oysters as reported in Samain et al. (2007). This supports the hypothesis that the energy expended during reproduction compromises the immune status of oysters, leaving them easily subject to mortality if stress occurs in postspawning stage (Li et al., 2007). In our study, the risk factor is obviously the presence of OsHV-1 in asymptotic oysters during the prespawning stage. As soon as the spawn occurred in the AC oysters, OsHV-1 proliferation may have occurred extremely rapidly as reported by Schikorski et al. (2011a), leading to massive mortality. This suggests that resistance to OsHV-1 infection is not related to the amount of energetic reserves, which is in the same direction that results found by Pernet et al. (2012) with similar concentration of carbohydrate and triacylglycerols for non-infected and infected *C. gigas*. It is also the first time that mortality due to OsHV-1 was reported in adults while animals were survivors of the disease occurring during their first year contrasting with results in Dégremont et al. (2010c). We easily explained it through the confined environment applied during the cohabitation trials. This characteristic allowed higher concentration of viral particles in the tank and lasted beyond the threshold of resistance of some unselected oysters, leading to mortality, while these oysters would have survived in field conditions.

The second result concerned the horizontal transmission of OsHV-1 from asymptomatic AC oysters to naïve J oysters in the unheated condition. Our study is the first to report such result in *C. gigas* under a sub-clinical status while several studies showed horizontal transmission of OsHV-1 from experimentally infected oysters during their productive infection to uninfected oysters (Burge and Friedman, 2012; Schikorski et al. 2011a). It should be noted that AC batch did not display mortality for the same period. We suggest the hypothesis that it might have been possible due to the seawater temperature pattern two weeks before and two weeks after the onset of the mortality, ranged from 14 to 17 °C (Fig. 2), combined to the flow-through seawater system, leading to slow OsHV-1 replication and low viral concentration in the tank.

Additionally, the third result showed faster and higher mortalities for the J oysters in the heated conditions at 22 °C than in the unheated conditions at 14–16 °C (Fig. 4(A), (B)) when those were grown along with the AC oysters. Acute mortality was also observed for the AC oysters for the within batch cohabitation trials (Fig. 3(A), (B)) while the temperature was 22 °C (Fig. 2). The kinetics of the mortality due to OsHV-1 in our study at 22 °C are in agreement to results found in field conditions (Dégremont, 2011; Pernet et al., 2012) or in other cohabitation trials in laboratory (Dégremont et al., 2010a; Schikorski et al., 2011a). Altogether, these results highlighted the strong influence of the seawater temperature as a risk factor on the viral replication and disease expression as also previously described in larvae (Le Deuff et al., 1996).

Moreover, a fourth result focused on the onset of the mortality and its kinetics related to the variation of the seawater temperature, especially for the cohabitation trial between the AC and J oysters. Thus, mortality started 10 days earlier in the unheated condition than in the heated condition (Figs. 2, 4(A), (B)). We proposed the hypothesis that OsHV-1 μVar replication was activated by a thermal stress through the variation of the temperature in the unheated condition. Additionally, the onset of the mortality for the within cohabitation trial for the AC oysters was later than for the cohabitation trial between the AC and J oysters. We suggested that mixed different batches induce additional stress, which promotes disease expression in infected susceptible animals.

Finally, the last result focused on the onset of the mortality for the cohabitation trial between the AC and J batches for the heated condition and for the cohabitation trial within batch for the AC oysters, but related to extern mechanisms. It is unclear to explain the onset of the mortality so late while seawater temperature was in favor to the disease, especially for the heated conditions (Fig. 2). Interestingly, two mortality outbreaks related to OsHV-1 were observed in the field in front of our laboratory (data not showed). The first occurred on May 12th and was much lower than the second which occurred on May 31st. These two mortality events preceded the mortality observed on May 17th for the AC and J oysters for the between batches cohabitation trial in heated condition and on June 4–7th for the AC oysters in the within batch cohabitation trial in both seawater conditions. We suggest that there may also some unknown molecules carried by the seawater should be capable to reactivate the virus in asymptomatic oysters. Such molecules could be viral Light particles (L-particles-like, without capsid and DNA), as described in moribund oysters infected with OsHV-1 μVar (Schikorski et al., 2011b). L-particles could be released in seawater since they can be found extracellularly in other host (Aleman et al., 2003), spreading and then contaminating the tanks. According to such hypothesis, those molecules were not destroyed by UV but were not capable to induce mortality as any J oysters dyed during the within batch cohabitation trials. Further investigations are needed to explore this hypothesis.

In conclusion, horizontal transmission of OsHV-1 between stocks depends on their genetic background. Selected OsHV-1 resistant oysters had lower mortality, lower prevalence, and so, lower role of reservoir, and limit the transmission of the disease than of unselected oysters. Horizontal transmission of OsHV-1 was also observed between asymptomatic and unselected adults to unselected juveniles under the tested conditions. Mortality due to OsHV-1 depended of the seawater temperature, which appeared as a strong risk factor that can reactivate the virus from latent-like or sub-clinical infection in asymptomatic adults. This finding is very important for epidemiological transmission and spread of the disease. This work brings

evidence to suggest a latent period where the virus can be detected in healthy or asymptomatic oysters but further work needs to be conducted in order to better understand the role of sub-clinical infection.

## Acknowledgments

We want to thank the hatchery, nursery and genetic teams of the Laboratory Genetics and Pathology, IFREMER La Tremblade and IFREMER Bouin, for their assistance in oyster production, as well as Jean Luc Seugnet for his help in field operations. Finally, we really thank Dr James Munro for his English corrections and comments of this manuscript.

## References

Aleman, N., Quiroga, M., Lopez-Pena, M., Vazquez, S., Guerriero, F., Nieto, J., 2003. L-particle production during primary replication of pseudorabies virus in the nasal mucosa of swine. Journal of Virology 77, 5657–5667.

Arzul, I., Renault, T., Thebault, A., Gerard, A., 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. Virus Research 84, 151–160.

Burge, C.A., Friedman, C.S., 2012. Quantifying ostreid herpesvirus (OsHV-1) genome copies and expression during transmission. Microbial Ecology 63, 596–604.

Dégremont, L., 2003. Etude des bases génétiques de la mortalité estivale et des relations avec la croissance chez les juvéniles de *Crassostrea gigas*. Université de Caen Basse Normandie, Caen 333.

Dégremont, L., 2011. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture 317, 94–98.

Dégremont, L., Ernande, B., Bédier, E., Boudry, P., 2007. Summer mortality of hatchery-produced Pacific oyster spat (*Crassostrea gigas*). I. Estimation of genetic parameters for survival and growth. Aquaculture 262, 41–53.

Dégremont, L., Soletchnik, P., Boudry, P., 2010a. Summer mortality of selected juvenile Pacific oyster *Crassostrea gigas* under laboratory conditions and in comparison with field performance. Journal of Shellfish Research 29, 847–856.

Dégremont, L., Bédier, E., Boudry, P., 2010b. Summer mortality of hatchery-produced Pacific oyster spat (*Crassostrea gigas*). II. Response to selection for survival and its influence on growth and yield. Aquaculture 299, 21–29.

Dégremont, L., Boudry, P., Ropert, M., Samain, J.-F., Bédier, E., Soletchnik, P., 2010c. Effects of age and environment on survival of summer mortality by two selected groups of the Pacific oyster *Crassostrea gigas*. Aquaculture 299, 44–50.

Ford, S.E., Xu, Z., Debrosse, G., 2001. Use of particle filtration and UV irradiation to prevent infection by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) in hatchery-reared larval and juvenile oysters. Aquaculture 194, 37–49.

Garcia, C., Thebault, A., Degremont, L., Arzul, I., Miossec, L., Robert, M., Chollet, B., Francois, C., Joly, J.-P., Ferrand, S., Kerdudou, N., Renault, T., 2011. Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Veterinary Research 42, 73.

Gay, M., Renault, T., Pons, A., LeRoux, F., 2004. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. Diseases of Aquatic Organisms 62, 65–74.

Le Deuff, R.-M., Nicolas, J.-L., Renault, T., Cochennec, N., 1994. Experimental transmission of a Herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. Bulletin of the European Association of Fish Pathologists 14, 69–72.

Le Deuff, R.-M., Renault, T., Gerard, A., 1996. Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. Diseases of Aquatic Organisms 24, 149–157.

Le Roux, F., Zouine, M., Chakroun, N., Binesse, J., Saulnier, D., Bouchier, C., Zidane, N., Ma, L., Rusniok, C., Lajus, A., Buchrieser, C., Medigue, C., Polz, M.F., Mazel, D., 2009. Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. Environmental Microbiology 11 (8), 1959–1970.

Li, Y., Qin, J.G., Abbott, C.A., Li, X.X., Benkendorff, K., 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. American Journal of Physiology — Regulatory, Integrative and Comparative Physiology 293, R2353–R2362.

Littell, R.C., Stroup, W.W., Freund, R.J., 2002. SAS® for Linear Models, Fourth edition. SAS Institute Inc., Cary, NC.

Oden, E., Martenot, C., Berthaux, M., Travaillé, E., Malas, J.P., Houssin, M., 2011. Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: determination of a viral load threshold to prevent summer mortalities. Aquaculture 317, 27–31.

Pepin, J.F., Riou, A., Renault, T., 2008. Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. Journal of Virological Methods 149, 269–276.

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pépin, J.F., Keck, N., 2012. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquaculture Environment Interactions 2, 215–237.

Renault, T., Novoa, B., 2004. Viruses infecting bivalve molluscs. Aquatic Living Resources 17, 397–409.

Renault, T., Ledeuff, R.M., Cochennec, N., Maffart, P., 1994. Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France — comparative study. Revue De Medecine Veterinaire 145, 735–742.

Samain, J.F., Degremont, L., Soletchnik, P., Haure, J., Bedier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J.L., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., Boudry, P., 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture 268, 227–243.

Saulnier, D., De Decker, S., Haffner, P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. Journal of Microbiological Methods 77, 191–197.

Sauvage, C., Pepin, J.F., Lapegue, S., Boudry, P., Renault, T., 2009. Ostreid herpes virus 1 infection in families of the Pacific oyster, Crassostrea gigas, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR. Virus Research 142 (1–2), 181–187.

Schikorski, D., Faury, N., Pepin, J.F., Saulnier, D., Tourbiez, D., Renault, T., 2011a. Experimental ostreid herpesvirus 1 infection of the Pacific oyster Crassostrea gigas: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus Research 155, 28–34.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., Pepin, J.-F., 2011b. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Veterinary Research 42, 27.

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Research 153, 92–99.

Jaber, Tareq, 2009. Analysis of alpha-herpesvirus genes that encode latency associated transcripts. ETD collection for University of Nebraska — Lincoln.

Webb, S.C., Fidler, A., Renault, T., 2007. Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): application in a survey of New Zealand molluscs. Aquaculture 272, 126–139.