Diseases of aquatic organisms
August 2014, Volume 110, Issue 3, Pages 201-211
http://dx.doi.org/10.3354:dao02756

Rearing practices identified as risk factors for ostreid herpesvirus 1 (OsHV-1) infection in Pacific oyster *Crassostrea gigas* spat

Julien Normand[1, 2, *], Jean-Louis Blin[1, 3], Aude Jouaux[1, 4, 5]

[1] Centre de Référence sur l'Huître, Université de Caen Basse Normandie, Caen, France
[2] IFREMER LERN Port en Bessin, Station de Port-en-Bessin, Avenue Général de Gaulle, 14520 Port-en-Bessin, France
[3] Synergie Mer Et Littoral, Zone conchylicole, 50 560 Blainville sur mer, France
[4] Université de Caen Basse-Normandie, 'Biologie des Organismes Marins et des Ecosystèmes Associés' (BioMEA), IBFA, SFR ICORE, 14032 Caen Cedex, France
[5] CNRS INEE, BioMEA, Caen, France

*: Corresponding author : Julien Normand, email address : julien.normand@ifremer.fr

Abstract:

Early detection of Pacific oyster spat infected with ostreid herpesvirus 1 (OsHV-1) could prevent introduction of OsHV-1-infected individuals into farming areas or onshore rearing facilities, thus reducing the risk of infection of naïve oysters in such production systems. Experiments were conducted on several hundred oyster spat provided by producers in order to examine whether early rearing practices could be considered as potential risk factors for (1) OsHV-1 infection as detected by molecular methods and (2) spat mortality experimentally induced through thermal challenge. Spat groups collected on oyster beds and hatchery spat reared in growout areas during summer exhibited higher viral DNA contamination and mortalities during the trial than spat kept in onshore rearing facilities. Quantification of viral DNA before and during the trial showed that infection prevalence and intensity changed over time and revealed latent infection initially unsuspected in 3 of 10 groups. Thermal challenge induced a clear increase in the probability of detecting infected individuals, particularly for groups exhibiting significant prevalence of OsHV-1-contaminated spat prior to the challenge. The use of detection methods are discussed in relation to early rearing practices and disease control strategies.

Keywords: Oyster spat ; Epidemiology ; OsHV-1 ; qPCR ; Thermal challenge

I. Introduction

To our knowledge, mortalities of Pacific oysters, *Crassostrea gigas*, were first reported in the middle of the 20th century. Summer mortality events were first noted on the west coast of USA in the late 1950s (Cheney et al. 2000), in Japan in 1961 (Mori 1979), in México since 1997 (Chávez-Villalba et al. 2007) and in France for the last 20 years (Samain et al. 2007). Until 2008, mortality outbreaks observed in Pacific oyster stocks in France were a relatively scarce phenomenon, having a complex etiology involving host, environment and pathogen (Samain et al. 2007). Along with environmental and oyster factors, several pathogens were found associated with these mortality events, including bacteria belonging to the *Vibrio* genus (Saulnier et al. 2010) and an ostreid herpes-like virus (Renault et al. 1994, 2000a). The first observation of herpes-like virus particles in oysters was by Farley et al. in 1972, during a mortality outbreak of *C. virginica*, associated with elevated-temperature effluent from a power plant in US. In France, the association between a herpes-like virus and oyster mortality was reported for the first time in the early 1990s in hatchery-reared *C. gigas* larvae (Nicolas et al. 1992). Molecular studies then allowed to characterize the viral genome and to confirm that the virus, named *Ostreid herpesvirus 1* (OsHV-1), belongs to the *Malacoherpesviridae* family (Davison et al. 2009).

In 2008, heavy mortalities hit Pacific oyster spat in all French production sites during the spring and summer (Dégremont 2011, Pernet et al. 2010, 2012), resulting in 50 to 100% spat losses depending on site, origin of spat and rearing practices. The death rate declined over winter, but mortalities recommenced in spring 2009 and have continued until 2013. A new variant of OsHV-1 emerged as a dominant genotype in association with the rapid increase of mortality in 2008, suggesting the emergence of a novel oyster disease (Segarra et al. 2010, Renault et al. 2012). Experimental challenges, made via the injection of viral particles or cohabitation of healthy oysters with symptomatic individuals, confirmed the high pathogenicity of OsHV-1 µVar (Schikorski et al. 2011a & 2011b, Y. He et al. unpubl.) which is now considered the most probable causative agent of spat mortalities in France. Severe disease events associated with OsHV-1 were also reported in 2009 in Ireland (Peeler et al. 2012) and 2010 in Australia and New-Zealand (Jenkins et al. 2013), suggesting that this virus is becoming a global problem for Pacific oyster culture.

To face this threat, producers and other stakeholders soon made clear their desire to reduce the spread of the disease. As there are no effective vaccines or treatments, health

68 management strategies were based primarily on risk reduction through the restriction of
69 oyster stock transfers between sites during summer months, at both regional and national
70 scales (Murray et al. 2012). In parallel, different research teams intensified their efforts to
71 gain knowledge of epidemiological aspects of oyster spat mortality (Peeler et al. 2012), and
72 to develop more accurate and cost-effective molecular tools for the detection of infected
73 individuals (Martenot et al. 2010). These tools are now routinely employed for the early
74 detection of infectious sources, aiming at excluding OsHV-1 infected spat from growout
75 areas to reduce infection risk.
76 Several methods are now available to detect OsHV-1 or to estimate viral loads in biological
77 samples: light microscopy and electron microscopy (Renault et al. 1994), and molecular
78 methods such as *in situ* hybridization (Arzul et al. 2002), PCR (Renault et al. 2000b) and qPCR
79 (Pepin et al. 2008, Martenot et al. 2010). A novel method based on thermal challenge in the
80 lab was also developed to detect latent infections. Thermal challenge consisted of exposing
81 a sub-sample of previously infected oysters to conditions that mimick field conditions in
82 which mortality events occur. The method was inspired by previous work that identified
83 temperature as a trigger for virus replication (Burge et al. 2006) and was first described in
84 Petton et al. (2013). This protocol is currently considered the standard method for revealing
85 latent infection in asymptomatic spat, although it could appear less specific than classical
86 diagnostic tools based on molecular methods due to the fact that temperature could also
87 trigger the replication of other pathogens (Petton et al. 2013).
88 Early detection of OsHV-1 is challenging. As with other members of *Herpesvirales* order
89 (Jones 2003), this virus may persist in its host after the primary infection, entering into a
90 latency stage (Arzul et al. 2002). The seasonality of mortality outbreaks in spat and virus
91 detection in relation with seawater temperature variation also suggests that OsHV-1 is able
92 to reactivate from latency and that this is triggered by temperature increase (Le Deuff et al.
93 1996, Burge et al. 2006, Oden et al. 2011). The identification of several OsHV-1 genes
94 involved in latency maintenance or in the lytic phase (Jouaux et al. submitted) strongly
95 supports this hypothesis.
96 In this paper, we report an experimental study conducted to determine the evolution of
97 OsHV-1-infection in different spat populations, in relation to potential risk factors. We
98 employed different batches of young oysters (<1 year) from various origins and histories.
99 Three methods for detection of early OsHV-1 infections were investigated: 1) qPCR

immediately upon delivery of the spat, 2) mortality in thermal challenge and 3) qPCR during thermal challenge.

II. Materials and Methods

II.1. Biological material and indicators of early rearing practices

Ten groups of oyster spat were obtained from oyster growers during the winter and early spring of 2012. For each group, information was collected about the origin of spat (produced in hatchery or collected on natural beds along the shore), and their potential exposure to OsHV-1 contamination, particularly during summer when mortalities occurred (Table 1).

As currently practiced, size-selective culling commonly induces high variability of spat size among groups. Knowing that physiological functions (such as food assimilation, oxygen consumption, subsequent growth and reproductive effort) are size-dependant in oyster (Pouvreau et al. 2006), we also measured mean individual mass and standard deviation by weighing 30 randomly selected spat in each population.

Table 1: Origin, history and individual mass (mean ± SD) for Pacific oyster *Crassostrea gigas* spat groups used in this study. NSC: natural spat collection; exp: exposed to the intertidal area

Batch	Spat origin	Intertidal rearing	Intertidal rearing in summer	Individual mass (g)
1	NSC	Yes exp.	Yes exp.	0.40 ± 0.03
2	Hatchery	Yes exp.	Yes exp.	0.70 ± 0.01
3	Hatchery	No-not exposed	No-not exposed	0.64 ± 0.05
4	NSC.	Yes exp.	Yes exp.	0.19 ± 0.01
5	Hatchery	No-not exposed	No-not exposed	0.17 ± 0.01
6	Hatchery	Yes exp.	Yes exp.	0.21 ± 0.01
7	Hatchery	Yes exp.	No-not exposed	0.50 ± 0.02
8	NSC.	Yes exp.	Yes exp.	0.10 ± 0.01
9	Hatchery	Yes exp.	No-not exposed	0.31 ± 0.01
10	NSC	Yes exp.	Yes exp.	0.14 ± 0.01

II.2. Virus analysis

Initial prevalence of OsHV-1 was estimated with 96 individuals, randomly sampled in each group upon spat delivery. Prevalence of OsHV-1 during thermal challenge was estimated by sampling 15 living individuals per group (5 in each replicate of challenge tanks), 3 weeks after the beginning of the trial.

Genomic DNA from each spat sample was extracted individually on an EpMotion5075 automated pipetting system (Eppendorf) with the Nucleospin 96 Blood kit (Macherey Nagel) according to manufacturer's protocol. Quality and quantity of genomic DNA was estimated on Nanodrop 2000 spectrophotometer (Thermoscientific). Due to the high number of individuals analysed for each group and condition, samples could only been tested individually.

Quantifications of virus were based on the qPCR TaqMan protocol described by Martenot et al. (2010), using the same amplification protocol and primers. Following these authors, this particular quantification method offers the best guarantees for precision and quantification prediction, considering that standard precautions (such as cross-validation with alternative protocol and the use of various internal quantification calibrators) were taken during the development of the assay. Around 350 ng of DNA were added for PCR reaction. A five concentration level standard control was added in each plate in order to establish the standard curve, and a negative control was also included to test for potential contamination. Virus quantity was reported as viral genomic units (VGU) per nanogram of genomic DNA extracted (Schikorski et al. 2011a and 2011b, Burge & Friedman 2012). The detection limit for the qPCR assay was 50 VGU. The thermocycler used for qPCR analysis was CFX 96™ Real Time System C1000™ Thermal Cycler (Biorad).

II.3. Thermal challenge

Experimental facilities are very similar to the ones described in Petton et al. (2013), consisting of a flow-through rearing system supplied with 1-µm filtered seawater exposed to UV irradiation flowing at 100 ml min^{-1}, that is the current method used to prevent the entrance of viral particles in hatchery. During the 4-week trial, three replicates of 200 spat per group were placed in 10 L tanks receiving this flow-through water. Oysters were fed constantly ad libitum with *Chaetoceros calcitrans* (49 x 10^6 cells/spat/day) and maintained at

21°C +/- 0.7. Mortality was estimated at the end of the trial, by calculating the percentage of dead individuals compared with the total starting number of spat.

II.4. Statistical analyses

All statistical analyses were conducted with R software, using Epicalc (Chongsuvivatwong 2012) and MASS packages (Ripley et al. 2013). As a first step, we conducted a risk factor analysis considering the 3 following proxies for infection status as the outcomes: 1) frequency of OsHV-1 detection by qPCR on delivery of the spat, 2) mortality in thermal challenge and 3) frequency of OsHV-1 detection by qPCR during thermal challenge. We transformed the OsHV-1 qPCR values to produce a (binomial) variable as follow: viral detection was considered as positive when quantification of the amount of OsHV-1 DNA exceeded 0.1 VGU ng^{-1} DNA (that is the rounded-up nearest value of the detection limit [0.14 VGU ng^{-1}]) and negative when it was less.

We considered early rearing practices (origin of spat, rearing in the intertidal area, rearing in the intertidal area during summer) and mean individual mass as potential risk factors.

Due to the fact that oyster seed from “natural” spat collections settle in the intertidal area during the summer period, only oysters from hatcheries could have avoided exposure to virus when mortalities occurred, and “rearing in the intertidal area” appeared logically nested in the “origin” effect. Similarly, “rearing in the intertidal area during summer period” was nested within “rearing in the intertidal area” (Figure 1). The following analytical strategy was therefore employed:

1) The effect of origin was first tested by contrast between groups from hatchery or spat collection in the field, considering all the groups;
2) The effect of rearing in the intertidal area effect was tested within hatchery groups by contrasting exposed (reared in the intertidal area) and unexposed (never reared in the intertidal area) groups;
3) The effect of rearing in the intertidal area during summer was tested within groups reared in the intertidal area, by contrasting exposed (reared in the intertidal area during summer) and unexposed (not reared in the intertidal area during summer) groups.

To this end, we used logistic (univariate) regression to fit models and estimate relevant odds ratio (OR) and 95% confidence intervals (CI). For binary independent variables (e.g. origin of

spat, rearing in the intertidal, rearing in the intertidal during summer period), we tested for the significance of the potential risk factor using a Chi-squared test of independence, whereas a Wald-test was applied for the continuous variable, mean mass.

As a second step, we focused on the evolution of OsHV-1 prevalence and load in response to thermal challenge and early rearing practices. To this end, we transformed the qPCR values to produce a discrete "virus-load" class variable having 5 levels: class 1= 0 to 0.1 VGU per ng of oyster DNA, class 2= 0.1 to 1 VGU ng^{-1} DNA, class 3= 1 to 10 VGU ng^{-1} DNA, class 4= 10 to 100 VGU ng^{-1} DNA, class 5= 10 to 1000 VGU ng^{-1} DNA. We then employed ordinal logistic regression with proportional odds assumption (Ananth & Kleinbaum 1997) to model frequency-by-OsHV-1 class for each treatment (before or during the thermal challenge), origin and early rearing practices, using the same approach that we employed for risk factor analysis to model nested effects. We also tested for these effects on observed frequency-by-OsHV-1 classes using the Chi-squared test.

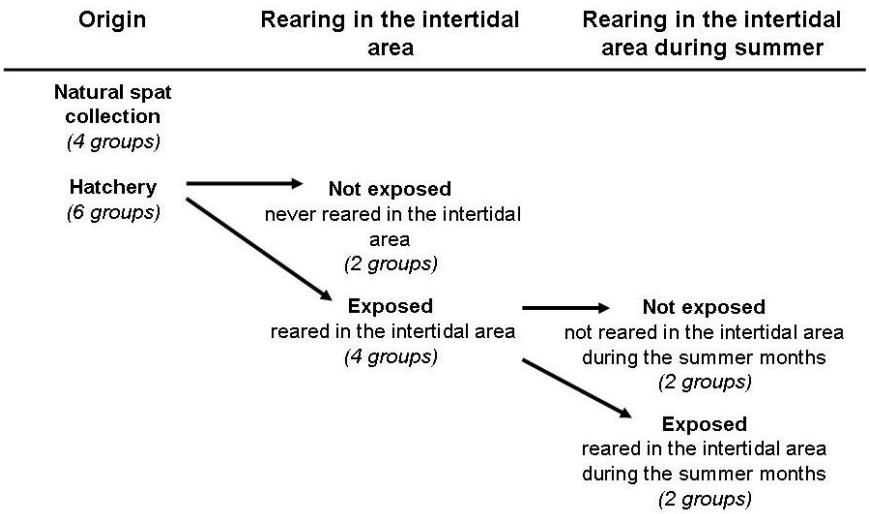


Figure 1: Origin of seed and rearing practices effects, with the number of spat groups used in this study.

III. Results

III.1. Virus detection on delivery of the spat

No OsHV-1 was detected in only 3 (Groups 3, 5 and 9; Table 1) of the 10 groups used in this study. Viral DNA was detected in a small fraction of the population in 4 groups: 3%, 9%, 1%,

4% in groups 2, 7, 8 and 10, respectively. The 3 remaining groups, 1, 4 and 6, had higher prevalences of: 24%, 19% and 24%, respectively (Figure 2a).

III.2. Mortality during thermal challenge

At the end of the experimental challenge, 6 groups had mean mortalities (among the 3 replicate tanks) greater than 20% (groups 1, 2, 4, 6, 8 and 10) and 2 of these (8 and 10) were greater than 70% (Figure 2b). Mortality rates in Group 8 ranged between 71% and 86%. For Group 10, however, mortality greater than 70% was observed for only one of the replicates, whereas the other two experienced mortality around 23% (Figure 2b). The 4 other groups (3, 5, 7, 9) had mortalities less than 5% at the conclusion of the thermal challenge (Figure 2b).

III.3. Virus detection during thermal challenge

OsHV-1 DNA was detected in every group tested during the thermal challenge (Figure 2c). Virus detection was more frequent in Groups 4, 6 and 8, ranging from 80% (12 cases positive among 15 samples) to 100%. Group 1 exhibited intermediate prevalence, with 33% (5/15) of the samples being positive. For the 6 remaining groups, OsHV-1 DNA was detected in only 2 to 3 individuals among the 15 tested (13 to 20%) (Figure 2c).

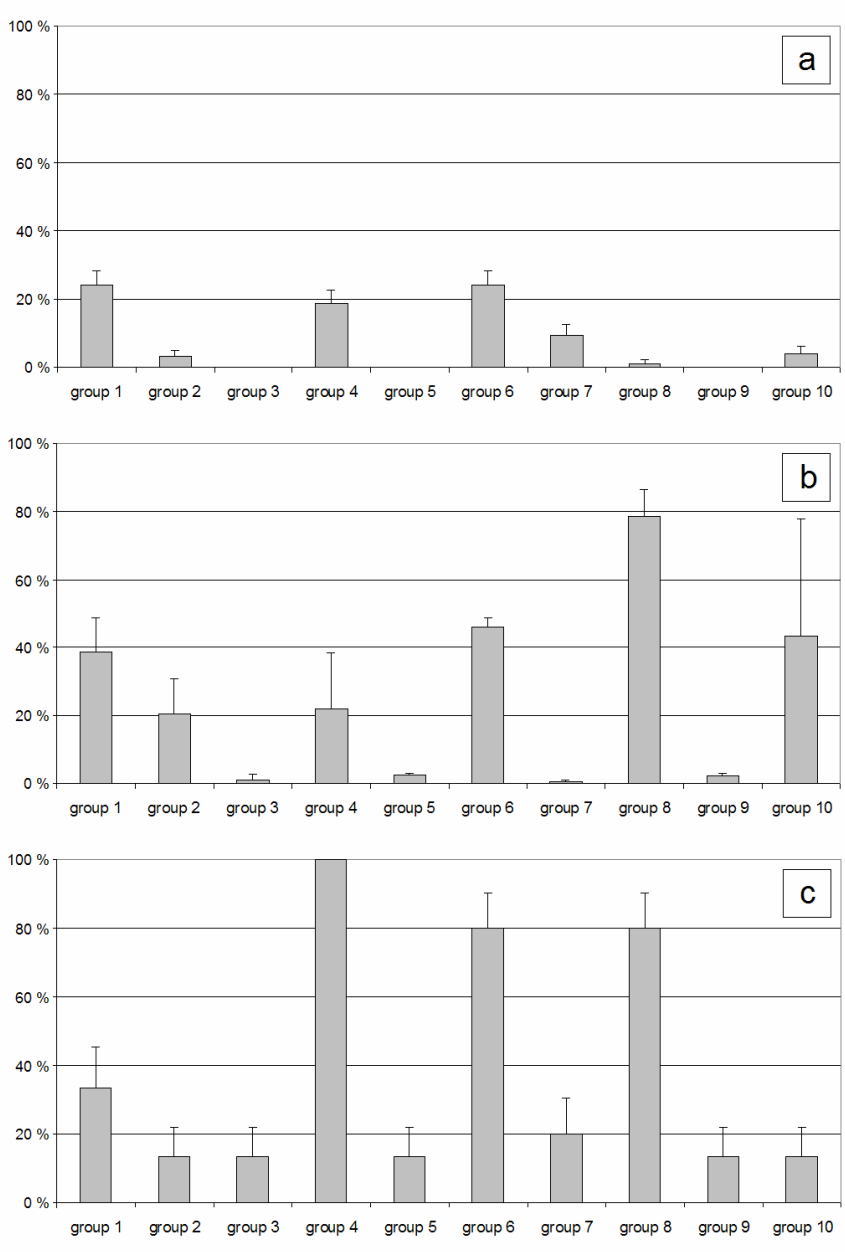


Figure 2: a) Proportion of spat OsHV-1 > 0.1 VGU ng^{-1} (in grey) among groups upon delivery of the spat (percentage and standard deviation of a proportion, n=96 individuals per group). b) Cumulative percent mortality among groups at the end (4 weeks) of thermal challenge (mean and standard deviation among tanks; n=200 individual per tank, 3 tanks per group). c) Proportion of spat OsHV-1 > 0.1 VGU ng^{-1} (in grey) among groups after 3 weeks of thermal challenge (percentage and standard deviation of a proportion, n=15 individuals per group).

III.4. Risk factor analysis

Rearing in the intertidal regions appeared to have a great influence on the probability of detecting OsHV-1 upon delivery of the spat (Table 2). Among the 2 groups 3 and 5 (96 spat

each) that were never reared in the intertidal area, no individual was found to be positive for virus. Individuals not exposed in the field during summer, or (to a lesser extent) coming from the hatchery, were also less likely to be positive for virus upon delivery (Table 2). The probability of detecting individuals infected by the virus increased from 2% to 14% when comparing hatchery-produced spat kept in on-shore facilities during summer versus those placed in the field. The Odds-Ratio (OR) for "rearing in the intertidal area during summer" effect was estimated to be 6.5 (95% CI, 2.9-16.1). In parallel, initial prevalence estimated by qPCR increased from 6 to 12% when comparing oyster seed from natural settlement instead of hatchery production, and the relevant OR was 2.1 (1.3-3.44) (Table 2).

Among the groups that were maintained in the hatchery during summer, mortalities during thermal challenge were less than 2%, suggesting that they were preserved from infection. Conversely, rearing in the intertidal area during summer, and, to a lesser extent, the use of natural spat collected in the field, sharply increased mortalities during thermal challenge (Table 2). For these two potential risk factors, estimated ORs were 32.9 (23.1-48) and 6 (5.3-6.9), respectively. Risk ratios indicated a 22.4-fold increase of the probability of dying during thermal challenge when hatchery seeds where reared in the intertidal area during summer, or an increase of 3.7-fold for spat from natural settlement compared with hatchery-produced spat.

The probability of detecting OsHV-1 during thermal challenge follows the same trend as that for mortality (Table 2). Mean prevalence estimated by qPCR was around 15% and did not appear to be significantly affected by rearing in the intertidal area, however rearing in this region during summer significantly increased the probability of detecting OsHV-1 from 15 to 47%, with a corresponding OR of 5 (1.6-15.4). Spat from natural recruitment were also more likely to be positive for the virus than hatchery seed. An estimated 6% of the oysters from hatcheries were found to be infected after 3 weeks of thermal challenge, whereas 12% of natural spat were. The OR for origin effect was estimated to be 3.8 (1.8-8.1).

Table 2: Effects of early rearing practices (origin, rearing in the intertidal area during summer, rearing in the intertidal area) on the probability of detecting OsHV-1 upon 1) spat delivery, 2) mortality during thermal challenge and 3) the probability of detecting OsHV-1 after thermal challenge. Chi-squared values and associated probabilities with exact odds ratios (OR) and 95% confidence intervals (CI).

		Origin (hatchery = 0)	Intertidal rearing during summer period (not exposed = 0)	Intertidal rearing (not exposed = 0)
OsHV-1 detection on spat delivery	Chi-squared / P-value	10.4 / 0.001	28.1 / <.0001	9.2 / <.0001
	OR (95% CI)	2.1 (1.3-3.44)	6.5 (2.9-16.1)	Infinite
Mortality during thermal challenge	Chi-squared / P-value	838.1 / <.0001	762.6 / <.0001	0.45 / 0.5
	OR (95% CI)	6 (5.3-6.9)	32.9 (23.1-48)	0.8 (0.4-1.6)
OsHV-1 detection during thermal challenge	Chi-squared / P-value	14.8 / <.0001	10.5 / 0.001	0.1 / 0.72
	OR (95% CI)	3.8 (1.8-8.1)	5 (1.6-15.4)	1.3 (0.2-7.3)

The effect of mean individual mass on OsHV-1 detection at the time of delivery of the groups appeared only marginally significant (P > 0.05) (Table 3), although the estimated coefficient from logistic regression roughly indicated that the probability of detecting virus tended to decrease with increased weight.

Mean individual mass appeared to have a significant protective effect on infection during thermal challenge. Estimated parameters of the model indicated that the log odds for the probability of mortality decreased by 4.43 for every gram increase in mass, with associated OR = 0.01 (0.01-0.02) (Table 3). In other words, an estimated 0.46 g mean individual mass appeared to protect the spat groups from mortalities superior to 10 % when challenged in our experimental conditions.

For OsHV-1 prevalence assessed during thermal challenge, the log odds for the probability detecting infected individuals also decreased by 4.20 for every gram increase in mass, and the OR was estimated to be 0.01 (0-0.12) (Table 3).

Table 3: Effects of mean individual mass (in grams) on 1) the probability of detecting OsHV-1 upon delivery of spat, 2) mortality during thermal challenge and 3) probability of detecting OsHV-1 during thermal challenge. Wald (Z) values and associated probabilities with exact odds ratios (OR) and 95% confidence intervals (CI).

		Mean	Standard deviation	Z	P-value
OsHV-1 detection on spat delivery	Intercept	-2.05	0.22	-9.4	< .0001
	Mean mass (slope)	-1.05	0.61	-1.7	0.0884
	OR (95% CI)	0.3 (0.1-1.2)			
Mortality during thermal challenge	Intercept	0.2	0.06	3.4	0.0007
	Mean mass (slope)	-4.43	0.2	-21.6	<.0001
	OR (95% CI)	0.01 (0.01-0.02)			

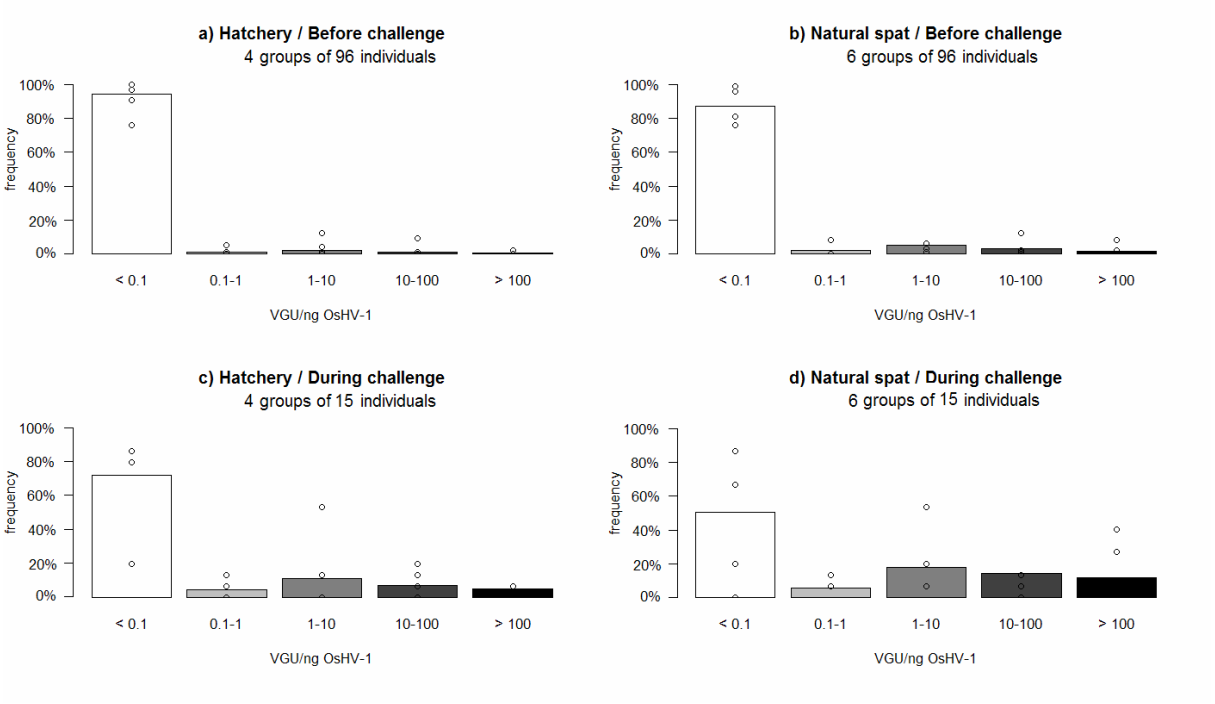
OsHV-1 detection during thermal challenge	Intercept	-0.8	0.34	2.4	0.0199
	Mean mass (slope)	-4.2	1.05	-3.99	<.0001
	OR (95% CI)	0.01 (0-0.12)			

282

283 III.5. Modeling of risk factors and thermal challenge effects on OsHV-1 infection and spread

284 When considering the entire dataset, highly significant effects were found both for origin
285 (Chi-squared= 31.82; p-value <.0001) and thermal challenge (Chi-squared= 111.72; p-value
286 <.0001) on ordinal frequency-by-OsHV-1 class outcome (Figure 3). When entering the trial,
287 groups of oysters from hatchery production exhibited higher frequencies of individuals in
288 which amounts of viral DNA did not exceed 0.1 VGU ng^{-1} (94% for hatchery groups versus
289 87% for natural spat groups) (Figure 3a and 3b). Thermal challenge resulted in a general
290 increase of the frequency-by-OsHV-1 classes greater than 0.1 VGU ng^{-1}, although heavily
291 infected individuals (> 1 VGU ng^{-1} DNA) were found more frequently in oyster spat from
292 natural spat collections than for those from hatchery production (44 versus 23%) (Figure 3c
293 and 3d).

294



295 Figure 3: Frequencies-by-OsHV-1 class (expressed in percentages) for origin and thermal
296 challenge effects. Bars represent estimated frequencies from the model, and circles
297 represent observed frequencies.

298

Within groups coming from hatchery production, highly significant effects were also found for exposure in the intertidal area (Chi-squared= 21.22; p-value= 0.0002) and challenge (Chi-squared= 37.67; p-value <.0001) on ordinal frequency-by-OsHV-1 class outcome (Figure 4). Before entering the thermal challenge, the detection of infected individuals was extremely low (around 1%) in groups kept away from the intertidal area whereas 9% of hatchery spat nurseried-cultured in the intertidal area exhibited more than 0.1 VGU ng^{-1} DNA (Figure 4a and 4b). The experimental trial resulted in a general increase in the prevalence of infected individuals in both groups, although infected oysters showing more than 1 VGU ng^{-1} DNA remained far scarcer in groups maintained in onshore facilities (5% versus 31%, respectively) (Figure 4c and 4d).

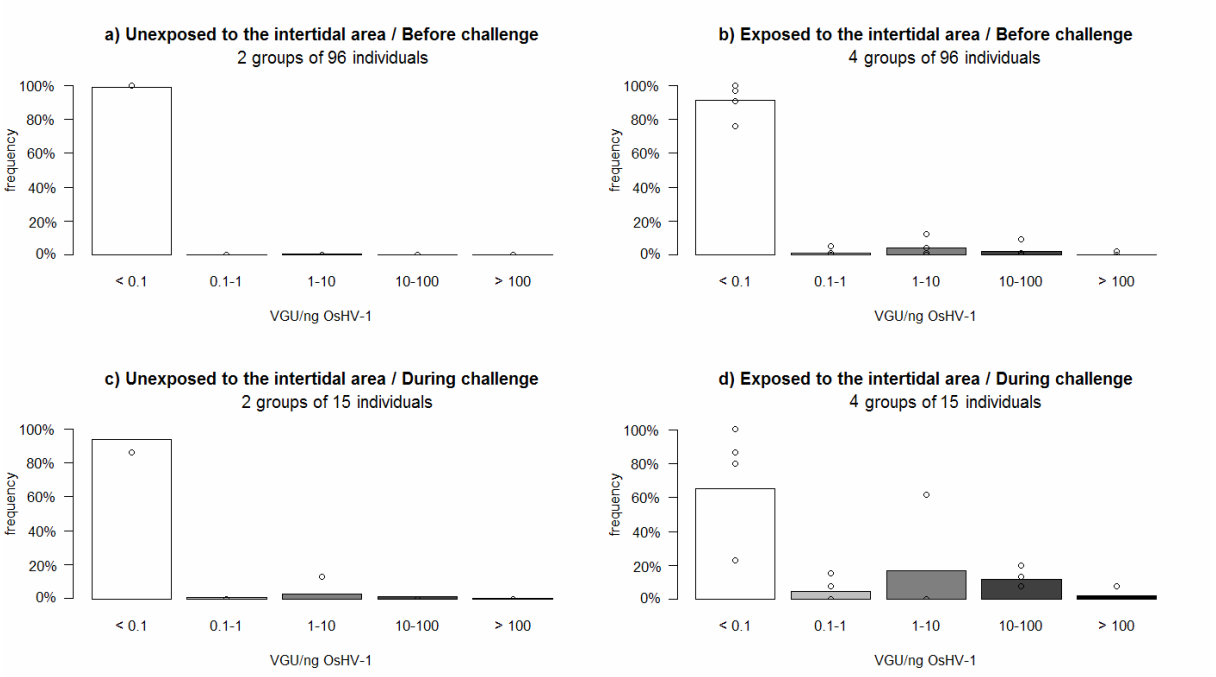


Figure 4: Frequency-by-OsHV-1 classes (expressed in percentages) for exposure to the intertidal area and thermal challenge effects, within hatchery groups. Bars represent estimated frequencies from the model, and circles represent observed frequencies.

Exposure to the intertidal area during the summer period (when oysters in nearby farms were dying with OsHV-1 infections) and thermal challenge were also found to have a highly significant effect on frequency-by-OsHV-1 class (respectively: Chi-squared= 87.03, p-value <.0001 and Chi-squared=25.48, p-value <.0001) (Figure 5). When entering the trial, only 5%

of oysters maintained in onshore facilities during the summer exhibited an amount of viral DNA greater than 0.1 VGU ng^{-1}, whereas this proportion reached 14% for spat reared in the intertidal area during this period (Figure 5a and 5b). Thermal challenge resulted in a general increase in the frequency-by-OsHV-1 classes greater than 0.1 VGU ng^{-1} DNA, although individuals exhibiting more than 1 VGU ng^{-1} were found more frequently in groups reared in the intertidal area during summer period (37% versus 15%) (Figure 5c and 5d).

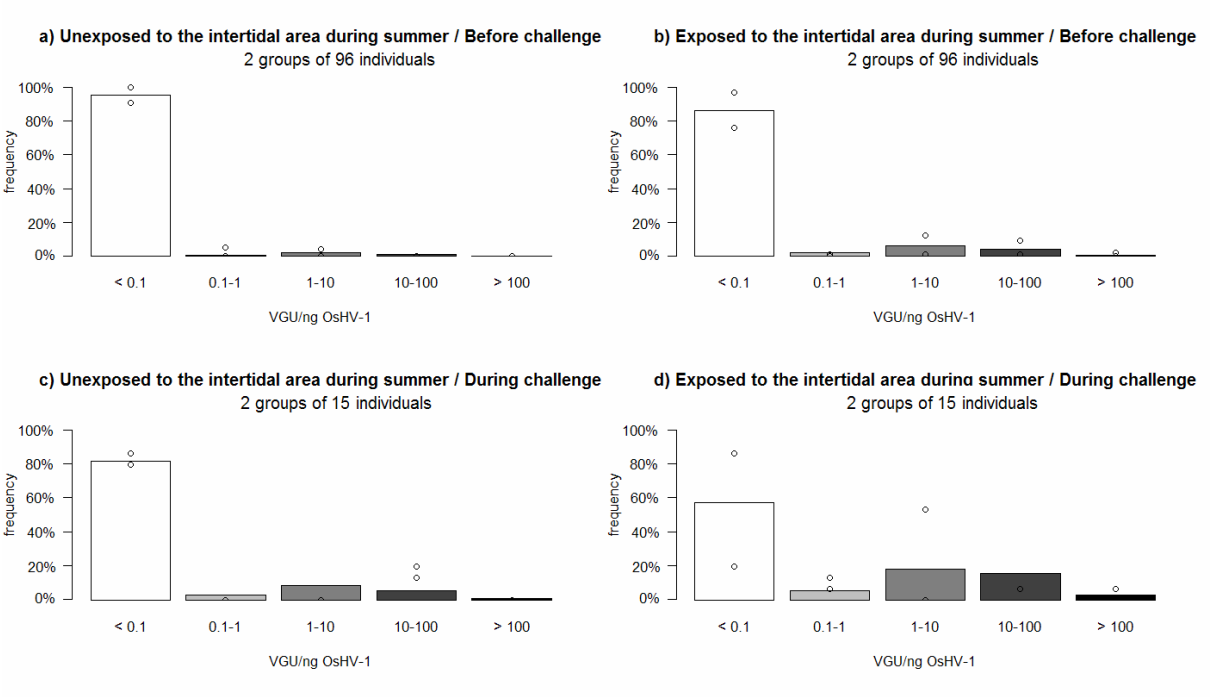


Figure 5: Frequency-by-OsHV-1 classes (expressed in percentages) for exposure to the intertidal area during summer and thermal challenge effects, within hatchery groups exposed to the intertidal area. Bars represent estimated frequencies from the model, and circles represent observed frequencies.

IV. Discussion

IV.1. Diagnostic methods

The 3 methods we used for the early detection of infection offered comprehensive characterization of infection status in seed groups. OsHV-1 detection through qPCR analysis upon delivery was frequent, detecting OsHV-1 DNA in 7 of 10 groups of spat considered in this study. In these groups, initial prevalence varied from 1 to 24%. This finding corroborates results from previous studies that also reported high degree of OsHV-1 contamination in

several groups of spat in France (Petton B., pers. comm.). Thermal challenge also induced mortalities greater than 20% in 6 of 10 groups. These mortalities led to losses that appeared clearly less than the mortality rates observed in cultivation areas, which usually range from 50 to 100%. This result is however consistent with the observations made by Petton et al. (2013), and is probably due to the fact that experimental trial did not fully re-create field conditions. As the dynamics of infectious diseases are typically related to the density of host populations (e.g. Krkošek 2010), we can hypothesize that relatively low densities as well as isolation from external virus sources limited the contagion and finally tempered the outbreak intensity in thermal challenge.

No OsHV-1-infected spat were found upon delivery in groups 3, 5 and 9. Conversely, groups 8 and 10, which initially exhibited only low prevalence of virus-infected individuals, experienced high mortality in the trial. These results suggest that the proportion of individuals having detectable viral infection on delivery was not the only factor involved in the expression of the disease during the thermal challenge. Knowing that OsHV-1 µVar established clearly as the dominant genotype since 2009 (Segarra et al. 2010, Renault et al. 2012), it appears either unlikely that the differences we observed in disease expression among groups were linked to differences in virulence for several virus strains. One can hypothesize that inter-group variation of resistance may be one of the other determinants of survival probability during the trial.

The thermal challenge trial did induce a clear increase of OsHV-1 detection frequencies in all tested groups. Some oysters were found to be positive for OsHV-1 even in groups 3, 5, 9 that appeared initially free from infected individuals, and did not exhibit significant mortalities during the trial. In these batches, virus prevalence during the thermal challenge was however found to be very low and the upper values for virus load only reached 10^3 VGU ng^{-1}. Similar results have been found in another study, more specifically dedicated to test early diagnostic tools (Petton B., pers. comm.). The coupling of thermal challenge with qPCR in the same diagnostic procedure, even though time-consuming and costly, appeared as the more effective methodology for the early detection of initially latent infections in spat groups.

IV.2. Risk factors and control strategy

Biosecurity in aquaculture should be based on early detection of contaminated stocks and infectious sources, in conjunction with management practices preventing later exposure to

pathogens (Lightner 2005). In France, cultivation practices are essentially based on rearing in open-water areas, and large-scale movements of oysters between cultivation areas are very common. In this context, biosecurity principles would initially appear to be very difficult to apply to the French oyster industry (Mohan et al. 2008, Peeler et al. 2012). Exclusion of OsHV-1 infected stocks was nevertheless proposed as a control strategy for spat mortalities, aiming at 2 different objectives. First, onshore rearing in hatcheries allows tight control of inputs. Entry of pathogens may therefore be prevented in such production systems by the strict application of biosecurity-based management practices. Second, limitation of the entry of heavily infected spat stocks in open-water rearing areas has also been envisaged as a potential control strategy, considering that massive or chronic exposure to OsHV-1 could constitute one aggravating factor for oyster seed mortalities.

In our study, OsHV-1 contamination of spat batches was found to be frequent, as viral DNA was detected in all 10 groups of oyster spat during the thermal challenge. This particular result was partially expected knowing that previous investigations also reported high prevalence of ostreid herpesvirus in asymptomatic older individuals (Arzul et al. 2002). It also suggests that strict exclusion of OsHV-1 infected stocks from production systems may require a substantial change in spat production practices, considering that viral contamination of spat groups appears to be currently widespread.

Conversely, risk factor analysis suggested that early rearing practices that limit exposure to the field also reduce the probability that OsHV-1 infection will subsequently develop if spat are kept away from external contamination. This finding was supported by only a limited number of observations (i.e. 10 groups), but is corroborated by the results of a larger-scale study currently in progress (Petton B., pers. comm.) that includes a larger number of spat groups. A negative correlation was also observed between weight and mortality, and between individual mass and viral detection. We could thus hypothesize that bigger oysters are more resistant to viral infection. Such a finding was partly expected, knowing that the establishment of genetically determined resistance to OsHV-1 depends from size or age (Dégremont, 2013).

In general, our results lead to the obvious conclusion that the use of large-sized spat from hatchery productions, nurseried in onshore facilities, would likely diminish the probability of disease outbreaks in closed production systems. The same recommendation could be made in order to limit the entry of OsHV-1 infected individuals into open-rearing areas, although

401 the effect of such a practice on mortality rates remains uncertain. In fact, the high
402 transmissibility of OsHV-1 (Schikorski et al. 2011a) coupled with the massive outbreaks
403 currently observed in oyster-farming areas (Pernet et al. 2012) should lead to the rapid
404 contamination of every spat batch deployed in the field, regardless of the initial prevalence
405 of OsHV-1 in the group. As for any other disease control strategy, such recommendations
406 should be tested in field trials before they are promoted as "better management practices",
407 offering the possibility of confirming the efficacy, as well as estimated costs and benefits
408 (Thurnbull et al. 2011).
409
410 IV.3. Effects of thermal challenge and risk factors on OsHV-1 infection and spread
411 Thermal challenge triggered the virus replication, characterized in all groups by a clear
412 decrease of the occurrence of individuals presenting less than 0.1 VGU ng^{-1} (and by a
413 resulting increase of the frequencies for all other OsHV-1 classes). During the trial, the OsHV-
414 1 DNA amounts per individual were found to vary over several orders of magnitude, even
415 among individuals from the same group (as an example, oysters from Group 8 exhibited
416 virus DNA amounts ranging from 0 to 10^5 VGU ng^{-1}). Such a result has, to our knowledge,
417 never been published before and supports our analytic approach, which considers
418 frequency-by-OsHV-1 classes rather than mean sample values. As a consequence of this high
419 inter-individual variability, the upper values reached here probably correspond to the mean
420 values for OsHV-1 DNA quantification at the height of an outbreak, reported in previous
421 studies (Schikorski et al. 2011a and 2011b), that ranged from 10^4 to 10^6 VGU ng^{-1}.
422 The latency-reactivation cycle has been extensively studied for herpesviruses infecting
423 vertebrates (Jones 2003), whereas only few studies to date have reported experimental
424 evidence for the reactivation of quiescent OsHV-1 infections in oysters. Our results showed
425 that the trial increased the frequency-by-OsHV-1 classes greater than 0.1 VGU ng^{-1},
426 suggesting that the increase of temperature and/or food availability triggered the
427 reactivation of latent viral infections (Burge et al. 2006). The evidence of virus reactivation
428 supports the hypothesis found in previous publications (Le Deuff et al. 1996, Arzul et al.
429 2002), which proposed that infected asymptomatic oysters probably play the role of carriers
430 and reservoirs of the virus, promoting OsHV-1 transmission to naïve individuals. This result
431 argues for reconsidering the recommendation of Oden et al. (2011) that proposed as a
432 control strategy the exclusion of infected groups based on mean individual amounts of viral

DNA. In fact, our data showed that the amount of OsHV-1 DNA measured at one point in time is clearly transient and that one oyster group could initially appear free of virus (below the limit of detection of one diagnostic tool) before revealing the disease when external stimuli induces viral proliferation.

From a theoretical standpoint, the infection process at the individual scale depends mainly on pathogenicity, which controls viral development, and healing processes related to the immunological response and resistance of the host (Hethcote 1989). At the group scale, however, the clear increase in the prevalence of heavily infected individuals observed during the trial probably resulted from internal proliferation occurring at the individual level and cross-contamination among oysters (Hethcote 1989). Moreover, mortalities occurring during the trial could eventually lead to the preferential elimination of heavily infected oysters, finally resulting in the under-representation of such classes of OsHV-1 infection. Due to the high number of processes affecting the resulting frequency-by-OshV-1 class distribution, our testing design clearly did not allow differentiating one effect from another. Such a goal will be pursued in subsequent studies, aimed at estimating epidemiological parameters and testing scenarios for disease spread using modeling.

Acknowledgements. The authors thank the producers for having provided the spat used in this study. They gratefully acknowledge the skilful assistance of the SMEL [Synergie Mer Littoral], UCBN [Université de Caen Basse Normandie] and Ifremer technical staff involved in oyster testing and molecular analyses. They also express their gratitude to Philippe Riou and Coralie Picoche who provided useful discussion and comments on earlier drafts of the manuscript. They also are especially grateful to Susan Ford for her help with editing the English.

Bibliography

Ananth CV, Kleinbaum DG (1997) Regression models for ordinal responses: a review of methods and applications. Int J Epidemiol 26(6): 1323-1333

464 Arzul I, Renault T, Thébault A, Gérard A (2002) Detection of oyster herpesvirus DNA and
465 proteins in asymptomatic *Crassostrea gigas* adults. Virus Res 84: 151-160
466
467 Burge CA, Griffin FJ, Friedman CS (2006) Mortality and herpesvirus infections of the Pacific
468 oyster *Crassostrea gigas* in Tomales Bay, California, USA. Dis Aquat Org 72: 31-43
469
470 Burge CA, Friedman CS (2012) Quantifying ostreid herpesvirus (OsHV-1) genome copies and
471 expression during transmission. Microb Ecol: 1-9
472
473 Chávez-Villalba J, Villelas-Avila R, Cáceres-Martínez C (2007) Reproduction, condition and
474 mortality of the Pacific oyster *Crassostrea gigas* (Thunberg) in Sonora, México. Aquaculture
475 Res 38(3): 268-278
476
477 Cheney DP, MacDonald BF, Elston RA (2000) Summer mortality of Pacific oysters,
478 *Crassostrea gigas* (Thunberg): Initial findings on multiple environmental stressors in Puget
479 Sound, Washington, 1998. J Shellfish Res 19(1): 353-359
480
481 Chongsuvivatwong V (2012) Epicalc: Epidemiological calculator. R package version 2.15.1.0
482 http://cran.r-project.org/web/packages/epicalc/ (accessed 23 Oct 2013)
483
484 Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B,
485 Studdert MJ, Thiry E (2009) The order Herpesvirales. Arch Virol 154: 171-177
486
487 Dégremont L (2011) Evidence for herpesvirus (OsHV-1) resistance in juvenile *Crassostrea*
488 *gigas* selected for high resistance to summer mortality phenomenon. Aquaculture 317: 94-
489 98
490
491 Dégremont L (2013) Size and genotype affect resistance to mortality caused by OsHV-1 in
492 *Crassostrea gigas.* Aquaculture 416-417: 129-134
493
494 Farley CA, Banfield VG, Kasnic GJ, Foster WS (1972) Oyster Herpes-type virus. Science 178:
495 759-760

He Y, Jouaux A, Ford S, Mathieu M, Lelong C, Guo X (in prep) Transcriptome response to OsHV-1.

Hethcote HW (1989) Three basic epidemiological models. In: Levins SA, Hallam TG, Gross L (eds): Applied mathematical ecology. Springer-Verlag Berlin/Heidelberg, Germany, p 119-144

Jenkins C, Hick P, Gabor M, Spiers Z, Fell SA, Gu X, Read A, Go J, Dove M, O'Connor W, Kirkland PD, Frances J (2013) Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis Aquat Org 105: 109-126

Jones C (2003) Herpes simplex virus Type 1 and bovine herpesvirus 1 Latency. Clin Microbiol Rev 16(1): 79-95

Jouaux A, Lafont M, Blin JL, Houssin M, Mathieu M, Lelong C (submitted) Physiological change under OsHV-1 contamination in Pacific oyster *Crassostrea gigas* through massive mortality events on fields. BMC Genomics submit 03.2013

Krkošek M (2010) Host density threshold and diseases control for fisheries and aquaculture. Aquacult Environ Interact 1: 21-32

Le Deuff RM, Renault T, Gérard A (1996) Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. Dis Aquat Org 24: 149-157

Lightner DV (2005) Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J World Aquac Soc 36(3): 229-248

526 Martenot C, Oden E, Travaille E, Malas JP, Houssin M (2010) Comparison of two real-time
527 PCR methods for detection of ostreid herpesvirus 1 in the pacific oyster *Crassostrea gigas*. J
528 Virol Methods 170 (1-2): 86-89
529
530 Mohan CV, Philips MJ, Bhat BV, Umesh NR, Padiyar PA (2008) Farm-levels plans and
531 husbandry measures for aquatic animal diseases emergencies. Rev sci tech Off Int Epiz 27(1):
532 161-173
533
534 Mori K (1979) Effects of artificial eutrophication on the metabolism of the Japanese oyster
535 *Crassostrea gigas*. Mar Biol 53: 361-369
536
537 Murray AG, Marcos-Lopez M, Collet B, Munro LA (2012) A review of the risk posed to
538 Scottish mollusc aquaculture from Bonamia, Marteilia and oyster herpes virus. Aquaculture
539 370-371: 7-13
540
541 Nicolas JL, Comps M, Cochennec N (1992) Herpes-like virus infecting Pacific oyster larvae,
542 *Crassostrea gigas*. Bull Eur Assoc Fish Pathol 12: 11-13
543
544 Oden E, Martenot C, Travaille E, Malas JP, Houssin M (2011) Quantification of Ostreid
545 herpesvirus (OsHV-1) in *Crassostrea gigas* by real time PCR: determination of a viral load
546 threshold to prevent summer mortalities. Aquaculture 317: 27-31
547
548 Peeler EJ, Reese AR, Cheslett DL, Geoghegan F, Power A, Thrush MA (2012) Investigation of
549 mortality in Pacific oysters associated with *Ostreid herpesvirus*-1 µVar in the Republic of
550 Ireland in 2009. Prev Vet Med 105: 136-143
551
552 Pepin JF, Riou A, Renault T (2008) Rapid and sensitive detection of ostreid herpesvirus 1 in
553 oyster samples by real-time PCR. J Virol Methods 149: 269–276
554
555 Pernet F, Barret J, Le Gall P, Corporeau C, Dégremont L, Lagarde F, Pépin JF, Keck N (2012)
556 Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with

557 farming practices in the Mediterranean Thau lagoo, France. Aquacult Environ Interact 2:
558 215-237
559
560 Pernet F, Barret J, Marty C, Moal J, Le Gall P, Boudry P (2010) Environmental anomalies,
561 energetic reserves and fatty acid modifications in oyster coincide with an exceptional
562 mortality event. Mar Ecol Prog Ser 401: 129-146
563
564 Petton B, Pernet F, Robert R, Boudry P (2013) Temperature influence on pathogen
565 transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*.
566 Aquacult Environ Interact 3: 257-273
567
568 Pouvreau S, Bourles Y, Lefebvre S, Gangnery A, Alunno-Bruscia M (2006) Application of a
569 dynamic energy budget model to the Pacific oyster, *Crassostrea gigas*, reared under various
570 environmental conditions. J Sea Res 56: 156-167
571
572 Renault T, Cochennec N, Le Deuff RM, Chollet B (1994) Herpes-like virus infecting Japanese
573 oyster (*Crassostrea gigas*) spat. Bull Eur Assoc Fish Pathol 14:64-66
574
575 Renault T, Le Deuff RM, Chollet B, Cochennec N, Gérard A (2000a) Concomitant herpes-like
576 virus infections in hatchery-reared larvae and nursery cultured spat *Crassostrea gigas* and
577 *Ostrea edulis*. Dis Aquat Org 42: 173-183
578
579 Renault T, Le Deuff RM, Lipart C, Delsert C (2000b) Developement of a PCR procedure for the
580 detection of a herpes-like virus infecting oysters in France. J Virol Meth 88: 41-50
581
582 Renault T, Moreau P, Faury N, Pepin JF, Ségarra A, Webb S (2012) Analysis of clinical ostreid
583 Herpesvirus 1 (*Malacoherpesviridae*) specimens by sequencing amplified fragments from
584 three virus genome area. J Virol 86(10): 5942
585
586 Ripley B, Venables B, Hornik K, Gebhardt A, Firth D (2013) MASS: function and data sets to
587 support Venables and Ripley 'Modern Applied Statiscs with S'. R package version 7.3-26
588 http://cran.r-project.org/web/packages/MASS/ (accessed 23 Oct 2013)

589

590 Samain JF, Dégremont L, Soletchnik P, Haure J, Bédier E, Ropert M, Moal J, Huvet A, Bacca H,
591 Van Wormhoudt A, Delaporte M, Costil K, Pouvreau S, Lambert C, Boulo V, Soudant P,
592 Nicolas JL, Le Roux F, Renault T, Gagnaire B, Géret F, Boutet I, Burgeot T, Boudry P (2007)
593 Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*)
594 and its relationship with physiological, immunological characteristics and infection process.
595 Aquaculture 268: 227-243

596

597 Saulnier D, De Decker S, Haffner P, Cobret L, Robert M, Garcia C (2010) A large-scale
598 epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and
599 correlation between virulence and Metalloprotease-like activity. Microb Ecol 59: 787-798

600

601 Schikorski D, Faury N, Pepin JF, Saulnier D, Tourbiez D, Renault T (2011a) Experimental
602 ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: Kinetics of virus DNA
603 detection by q-PCR in seawater and in oyster samples. Virus Res 155: 28–34

604

605 Schikorski D, Renault T, Saulnier D, Faury N, Moreau P, Pépin JF (2011b) Experimental
606 infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of
607 oyster spat susceptibility. Vet Res 42: 1-13

608

609 Segarra A, Pépin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of
610 a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of
611 Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res 153: 92–99

612

613 Thurnbull JF, Berril IK, Green DM, Kaye R, Morris D, Murray AG, del-Pozo J, Shinn A (2011)
614 Applied epidemiology with examples from UK aquaculture. Aquaculture Res 42: 21-27

615