Aquaculture International
December 2014, Volume 22 (6), Pages 1767-1781
http://dx.doi.org/10.1007/s10499-014-9781-7

Mortality associated with OsHV-1 in spat *Crassostrea gigas*: role of wild-caught spat in the horizontal transmission of the disease

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Abstract:

The French oyster production of *Crassostrea gigas* is based on two sources of spat: wild-caught (WC) and hatchery-produced (HP). Massive mortality related to the ostreid herpesvirus type 1 (OsHV-1) has affected both sources in France since 2008. We investigated the mortality in juvenile *C. gigas* due to the horizontal transmission of OsHV-1 within (separated condition) and between (mixed condition) the two spat sources in three environments from April to June 2010. In the separated condition, no mortality was observed in the HP batches, while the WC batches experienced moderate to high mortality (40–80 %). In contrast, the WC and HP batches experienced high mortality in all tested environments for the mixed condition. At the beginning of the trial, the HP batches were all negative for OsHV-1 DNA detection by real-time PCR, while the WC batches were all positive for OsHV-1 DNA detection by real-time PCR, even though the percentage of virus DNA-positive oysters and viral load were low. During the experiment, all batches that exhibited mortality were positive for OsHV-1 with a high viral load, while OsHV-1 was never detected for the HP batches of the separated condition. Together, our results demonstrated that OsHV-1 was horizontally transmitted from the WC oysters to the HP oysters. Our study is the first to indicate that the mortality related to OsHV-1 in HP oysters can be avoided using ponds or tanks. However, these oysters were always protected from OsHV-1, and HP oysters could also experience mortality and spread the disease similar to the WC oysters if such care is not used. Finally, the persistence of OsHV-1 at a sub-clinical level in certain oysters supports the hypothesis that the virus can be reactivated and cause viral replication. The use of the two spat sources is discussed to better understand the spread of the disease among oyster stocks.

Keywords: Crassostrea gigas ; Wild-caught ; Hatchery-produced ; Spat ; Mortality ; Horizontal transmission ; Ostreid herpesvirus OsHV-1

INTRODUCTION

The production of French *Crassostrea gigas* oysters is based on two sources of spat: wild-caught spat, especially from Marennes-Oléron Bay and Arcachon Bay, which are both located along the French Atlantic coast, and hatchery-produced spat from commercial hatcheries in France. The amount of hatchery-produced spat has increased regularly each year, reaching nearly 3 billion spat units in 2012. The increasing demand for hatchery-produced oysters is primarily driven by triploids. Moreover, the amount of wild-caught spat varies each year depending on disease and environmental conditions, as larvae and juveniles are highly susceptible to the Ostreid Herpesvirus type 1 (OsHV-1) (Le Deuff et al. 1994; Le Deuff et al. 1996; Renault et al. 2000; Dégremont 2011). Since 2008, disease investigations have revealed the involvement of OsHV-1 in numerous cases of recurrent and massive mortality that have been reported in juvenile *C. gigas* in Europe, Australia, New Zealand and on the western coast of the USA (EFSA 2010; Segarra et al. 2010; Cameron and Crane, 2011; Garcia et al. 2011; Burge and Friedman 2012; Lynch et al. 2012; Martenot et al. 2012; Peeler et al. 2012; Pernet et al. 2012; Roque et al. 2012; Jenkins et al. 2013; Paul-Pont et al. 2013). Similar mortality is also expected in larvae in the wild but has not yet been documented. To address the massive mortality related to OsHV-1, two strategies have been developed to supply more spat to the French oyster industry. The first has been initiated by hatcheries that develop a breeding program for OsHV-1-resistance in *C. gigas* oysters, because this trait could be selected (Dégremont et al. 2010b; Dégremont 2011). The second simply increases the number of spat produced by the commercial hatcheries or those caught in the wild by oyster farmers who have increased the capacities for spat collection. The

latter strategy results in more wild and unselected spat being caught, and this spat could potentially be infected with OsHV-1.

The transfer of oysters between the growing areas has been stipulated as a major source of disease transmission in France and throughout Europe. To better understand the oyster production cycle in France, note that wild-caught spat settle on various substrates during the summer, primarily along the Atlantic coasts. They are then detached the following winter and spring and sold to the oyster farmers. This process involves numerous oyster transfers as the spat are moved between growing areas (Goulletquer and Le Moine 2002). Meanwhile, hatchery-produced oysters are available throughout the year, and most of them are maintained either in a nursery or in field conditions before they are sold and then transferred between growing areas. Spat from both sources are then capable of spreading pathogens if they are grown in a contaminated area. Oysters can be produced in batches to prevent their exposure to the mortality risk factors in a controlled environment in the hatchery and nursery, as described in Dégremont et al. (2010b) and Dégremont (2011). These systems utilize UV filtration or seawater that has been pumped away from the oyster leases and stored for a certain amount of time in ponds. Lastly, transfers continue to occur throughout the life of the oysters as stocks are routinely moved within the local field grow-out sites, with sites dedicated to spat, juveniles or adults, as well as among coastal areas with numerous transfers of adults from Brittany or Normandy to the Charentais Sounds and Marennes-Oléron Bay, where most of the oysters are grown in ponds before being sold.

Every year since 2008, massive mortality outbreaks related to OsHV-1 have been observed in *C. gigas* spat. The peak of the disease usually occurs as soon as the seawater temperature exceeds 16°C, and other mortality events related to OsHV-1 may still be observed in

naïve juveniles when they are transferred to a contaminated area (Dégremont 2013). Therefore, the disease reoccurs the next spring in the new generation of hatchery-produced and wild-caught spat, regardless of the environmental conditions during the fall and winter, meaning that OsHV-1 remains persistent in asymptomatic *C. gigas* or other organisms, as also suggested by Arzul et al. (2002) and Peeler et al. (2012).

Two approaches were developed to better characterize the risk for the natural transmission of OsHV-1 among oyster stocks cultured in France: i) investigating the horizontal transmission of OsHV-1 between naïve hatchery-produced oysters and adult oysters that survive a mortality outbreak caused by the disease, as described in Dégremont et al. (2013), and ii) investigating the transmission between wild-caught and naïve hatchery-produced spat. This study reports the second approach. The primary objective of this study is to investigate the natural horizontal transmission of OsHV-1 throughout cohabitation trials within and between the two sources of spat using several batches per source in controlled conditions (laboratory) and in uncontrolled environments (ponds and grow-out field).

MATERIALS AND METHODS

Oyster batches

Two batches of wild-caught spat were purchased from oyster farmers in March 2010. One batch was from the Marennes-Oléron Bay, hereafter referred to as WC1, and the second one was from the Arcachon Bay, hereafter referred to as WC2. In France, these two bays account for

approximately 80% of the wild-caught production. The two batches were 9 months old, and no data were available with regard to their life history, specifically their cumulative mortality at reception.

Three hatchery-produced batches were used, including two diploids, hereafter referred to as HP1 and HP2, and one triploid, hereafter referred to as HP3. All of the HP batches were spawned at the Ifremer hatchery in La Tremblade in August 2009. For the diploid batches, the parents were sampled in the Marennes-Oléron Bay, and 6 and 7 males were crossed with 19 and 21 females to produce HP1 and HP2, respectively. For the triploid batch, the spermatozoa produced by 14 tetraploid males from the broodstock maintained at the Ifremer hatchery and used by the French commercial hatcheries to produce triploids, fertilized oocytes from the same 19 females used for HP1. The larvae were grown in 30-L tanks for 2-3 weeks. Competent larvae for metamorphosis were then settled in 120-L tanks using cultch. When the spat reached 2 mm, they were transferred to the Ifremer nursery in Bouin for intensive growth using raw seawater enriched with *Skeletonema costatum*. Lastly, the three hatchery-produced batches were transferred to La Tremblade for the survey, and any abnormal mortality was recorded from spawning to the beginning of the survey. At the beginning of the experiment, the individual weight of the oysters was similar among the five batches and was approximately 1 g.

Ploidy analyses

The DNA ploidy level of the juveniles was determined using flow cytometry (FC) with DAPI (4,6-Diamidino-2-phenylindole) staining. For each batch, 100 spat were randomly sampled and individually analyzed. The nuclei were extracted from small pieces (1 mm^2) of gills, mixed

with 2 µl of trout red blood cells (TRBC, Coulter DNA Reference Calibrator, 629972) as an internal standard solution, and stained with DAPI at a concentration of 2 µg/ml in a 2-ml final solution. FC was performed on a PA II flow cytometer (Partec) with the following conditions: excitation-100 W mercury lamp, UG 1 (290–410 nm, 3 mm), chromatic beam splitter (TK 420), emission-beam splitter (TK 420, TK 560), and barrier filter (CG 455) for the DAPI signals. The peak positions and the coefficients of variation (CV) were calculated automatically (PARTEC PAS II software package). At least 2000 nuclei were analyzed from each sample. The ratio between the fluorescence channels of the nuclei and the internal peaks is characteristic of the DNA ploidy level, which is equal to 0.4 for diploids and 0.6 for triploids.

Experimental design

The experiment was conducted from April 1st 2010 to June 30th 2010. Three sites in La Tremblade, all located within an area of 300 meters in diameter (N 45°47’55”; W 1°9’4”), were used for the survey: the Ifremer’s laboratory, the Ifremer’s ponds, and the field (Fig. 1). At each site, two temperature probes (Progesplus, 59780 Willems, France) recorded the seawater temperature every 30 minutes.

In the field, three bags per batch of 200 oysters each were attached to iron racks, which is the cultural practice used by most French oyster farmers (Table 1). Numerous oyster leases surround our experimental site.

In the ponds, each batch was grown alone in one pond with three bags containing 200 oysters each, which corresponds to the separated condition, *i.e.* the cohabitation trial among oysters within the batch. Another pond received three hatchery-produced batches and the wild-

caught batch WC1, which corresponds to the mixed condition, *i.e.* the cohabitation trial between batches. Unfortunately, the WC2 batch could not be tested in the mixed condition due to the lack of ponds. Three bags were used for the mixed condition, each containing four smaller bags of 50 oysters per batch, to obtain a total of 600 oysters in the pond as for the separated condition (Table 1). The ponds were naturally alimented by seawater during the spring tides when the tidal coefficient exceeds 85. The average depth of each pond was approximately 70 cm for a volume of 250 m^3.

The WC2 batch was not tested in the laboratory due to the lack of space. Three 50-L tanks were used per batch for the separated condition, each containing 200 oysters. For the mixed condition, three tanks containing 50 oysters per batch were used for the hatchery-produced batches and the WC1 batch, as in the pond condition. Flow-through seawater enriched with *Skeletonema costatum* was renewed hourly and constantly filtered and UV-treated to protect the oysters from the external mortality risks factors, particularly OsHV-1. All tanks were carefully cleaned twice per week.

Lastly, extreme care was used to avoid accidental contamination among the tanks or among the ponds by changing gloves or waders between each tank or pond. Unfortunately, one tank containing HP2 oysters was contaminated with the seawater of another tank containing WC1 oysters on April 30th, while abnormal mortality was recorded for the WC1 oysters. The HP2 oysters in this tank exhibited high mortality a few days later (90%) and were excluded from the studies.

Mortality survey

Dead and live oysters were counted once per week in the laboratory and pond conditions and every two weeks in the field condition until the end of the experiment on June 30th. Dead oysters were not removed, except for those sampled for the disease diagnoses.

OsHV-1 DNA detection and quantification

Since 2008, the disease diagnoses revealed that most of the mortality outbreaks reported in spat *C. gigas* are due to OsHV-1, and no other relevant pathogens were found (Guichard et al. 2011). Although *Vibrio splendidus* could be associated with mortality events in *C. gigas* (Lacoste et al. 2001; Le Roux et al. 2002), routine tools to discriminate virulent from non-virulent strains of *V. splendidus* are lacking. Furthermore, *V. splendidus* is more likely to be ubiquitous because correlations between the mortality and the presence or the bacterial load of *V. splendidus* were weak, negative and not significant in contrast to OsHV-1 during a mortality outbreak in juvenile *C. gigas* (Dégremont, 2011). Therefore, this research only examined OsHV-1 DNA.

At the beginning of the experiment, 50 oysters per batch were sampled for OsHV-1 detection and quantification. Additionally, 5 moribund oysters and 12 live oysters per batch, per site, and per condition were sampled during the mortality peaks and at the end of the experiment, respectively.

The OsHV-1 DNA was detected and quantified in each sampled oyster using a highly sensitive real-time PCR technique developed by Pépin et al. (2008). Briefly, DNA was extracted from 25 mg of fresh oyster tissue (mantle) using the QiagenQiamp® tissue mini kit, and 20 ng of DNA was used for the real-time PCR (Stratagene) with the following conditions: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and

72 °C for 45 s. The primer pairs used to detect the viral DNA were described in Webb et al. (2007) for targeting the OsHV-1 DNA polymerase sequence. All results were expressed as the viral DNA copy number per mg of oyster tissue.

Statistical analyses

The cumulative mortality on June 30th was analyzed per site using the GENMOD procedure SAS® software version 9 with a logit transformation and a binomial distribution. The logistic regression models used were the following:

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

where Y_{ij} is the probability of an unfavorable response (dead for the mortality) in the laboratory or the pond for the ith batch in the jth condition (mixed and separated), and μ is the intercept.

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

where Y_i is the probability of an unfavorable response in the field for the ith batch, and μ is the intercept.

Multiple comparisons were conducted using the least squares means statement and the SLICE option, which allows for the testing of the condition factor for each batch or the batch factor for each condition when a significant interaction is found between both factors. Thus, the statistical test is more powerful than rerunning the analysis within the batch because the degrees of freedom are not reduced (Littell et al. 2002).

RESULTS

DNA ploidy determination

FC measurements of the DNA ploidy level showed that all batches used in this study were effectively of the expected DNA ploidy level. All wild-caught spat (WC1 and WC2), as well as all spat from HP1 and HP2 were of the expected normal diploid ploidy levels. This characteristic was evidenced by the single peak and a ratio of fluorescence of 0.4, which is typical of diploid nuclei. Similarly, all HP3 spat were triploid, as evidenced by the single triploid peak and a ratio of fluorescence of 0.6.

Seawater temperature

The seawater temperatures at the three sites are shown in Fig. 2. The temperature ranged from 11 to 15°C at the beginning of the experiment and reached 21 to 27°C at the end of June. The seawater in the ponds was highly variable on April 22nd or May 4th due to the renewal of the seawater, which only occurs during the spring tide, combined with the weather conditions due to the small volume of the pond.

Mortality

In the field, batches HP2 and WC1 were the first to exhibit mortality at a low level (<30%) in May. During the first two weeks of June, massive mortality was reported for all

batches (Fig. 3). The mean mortality (± standard deviation) on June 30th was 67 ± 14%, ranging from 52 to 88% (Table 2). A significant difference in the mortality among the batches was found ($P < 0.01$), with the lowest mortality for the WC batches (54%), intermediate mortality for HP1 and HP3 (70%), and the highest mortality for HP2 (88%).

In the pond, no abnormal mortality was reported for the three HP batches throughout the experiment, while high mortality (39-82%) occurred for the two WC batches for the separated condition, primarily between April 29th and May 12th (Fig. 4a and Table 2). A major mortality outbreak (94%) was also observed during this period for all batches in the mixed condition (Fig. 4b). A significant interaction was found between the batches and the conditions ($P = 0.02$). At the batch level, the mortality was significantly higher for the mixed condition than for the separated condition for all batches except for WC1 (Table 2). At the condition level, a significant difference of mortality among the batches was only found for the separated condition ($P < 0.01$), with a lower mortality for the HP batches. The mixed condition did not show significant differences ($P = 0.67$).

Similarly, same results were found in the laboratory. Mortality was primarily observed during the first two weeks of May, but it only occurred for all replicates containing the WC1 for the separated condition (Fig. 5a). For the mixed condition and the same period, only one tank exhibited high mortality (81%), ranging from 57% for WC1 to 100% for HP2 (Fig. 5b). Because mortality was not observed for the two tanks in the mixed condition, the mean mortality for this condition dropped to 27%, and the standard deviation within batches was high. The batches and the condition showed a significant interaction ($P < 0.0001$). At the batch level, the mortality was always significantly higher in the mixed condition than in the separated condition except for the WC1. At the condition level, the mortality significantly differed among the batches of both

conditions, with a higher mortality for the WC1 batch in the separated condition and lower mortality for the WC1 batch in the mixed condition ($P < 0.0001$) (Table 2).

OsHV-1 DNA detection and quantification

At deployment, OsHV-1 DNA was not detected in any of the 150 HP oysters screened on April 1st, while 2% and 14% of the WC2 and WC1 oysters, respectively, were found to be positive but contain a low viral load ($< 10^{+4}$ DNA copy per mg of fresh tissue)(Table 3).

Irrespective of the site, condition or batch, all oysters sampled that were moribund during the mortality event were positive for a very high viral load, exceeding 10^{+6} DNA copies per mg of fresh oyster tissue.

At the endpoint, OsHV-1 was not detected in any of the HP batches for the separated condition in the laboratory or the pond on June 30th (Table 3). OsHV-1 was detected at a low viral load in 8% of the WC2 oysters in the pond as well as in 33% of the HP3 and 9% of the WC1 in the mixed condition in the laboratory. Lastly, a low level of OsHV-1 ($< 10^{+4}$ DNA copies per mg of fresh tissue) was also detected approximately 25% of the oysters in all batches from the field on June 30th.

DISCUSSION

The two types of spats (wild versus hatchery) used in this work were assumed to be representative of their respective origin in France. WC1 and WC2 originated from the Arcachon and Marennes-Oléron Bays, respectively, which account for 80% of the annual wild-caught spat

278 production in France on average. Additionally, the *C. gigas* populations in France were not
279 genetically differentiated (Rohfritsch et al., 2013). Alternatively, diploid HP batches were
280 produced using genitors sampled in the Marennes-Oléron Bay, while triploids were produced
281 using tetraploid males from the same broodstock used by the French commercial hatcheries. The
282 incidence and the kinetics of the mortality, combined with the detection of high viral loads (>
283 10^{+6} DNA copies per mg) in moribund oysters, strongly support that OsHV-1 was the main cause
284 of the mortality, as reported in previous studies (Pépin et al. 2008; Sauvage et al. 2009; Oden et
285 al. 2011; Dégremont et al. 2013). A high variation in mortality and in resistance to OsHV-1 is
286 common among *C. gigas* batches, as evidenced by Sauvage et al. (2009), Dégremont et al.
287 (2010c) and Dégremont (2011). This variation explains the variation in mortality observed
288 among the three HP batches in the present study in the field or in the mixed condition in the
289 laboratory (Figs. 3 and 5b). The main objective of this study was not to compare the HP batches,
290 but to study the horizontal transmission of OsHV-1 throughout cohabitation trials within batches
291 and between WC and HP batches.

292 Low and chronic mortality was observed in May for WC1 and HP2 in the field (Fig. 3).
293 This result could be easily explained by the activation of the replication of OsHV-1, which
294 started as soon as the temperature reached approximately 14 to 16°C, combined with a relatively
295 higher susceptibility of HP2 to viral infection compared to the other HP batches. The kinetics of
296 the mortality related to OsHV-1 according to the seawater temperature pattern agrees with the
297 results found in the cohabitation trial regarding transmission between infected adults and naïve
298 juvenile *C. gigas* (Dégremont et al. 2013). For instance, acute mortality was observed 10 to 12
299 days later when the temperature reached and remained above 16°C, regardless of the site where
300 the oysters were grown (Fig. 2), supporting the important role of the seawater temperature in

mortality related to OsHV-1 (Garcia et al. 2011). This value of the seawater temperature is in agreement with several studies that clearly showed the lower threshold of 16°C, beyond which disease transmission and mortality related to OsHV-1 occur (Dégremont 2013; Dégremont et al. 2013; Petton et al. 2013). The mortality event lasted over two weeks, and no other mortality event was reported until the end of the experiment (Figs. 3, 4 and 5), which is characteristic of the OsHV-1 mortality pattern.

The major findings of this study concern the role of the origin of the batch, *i.e.* hatchery-produced and wild-caught spat or contaminated and disease-free animals, on the transmission of the disease, as well as the possibility to grow oysters without mortality due to OsHV-1. At the beginning of the experiment, OsHV-1 was not detected in any of the 150 oysters screened among the three HP batches (diploids and triploids), which agrees with all of our OsHV-1 screening on HP oysters performed since 2009 (more than a 1000 individuals screened) (Dégremont 2011 & 2013; Dégremont et al. 2013). In contrast, both WC batches were found to be infected with OsHV-1, even though the prevalence and viral load were low. This finding indicates that WC oysters were infected in their respective native areas, where mortality related to OsHV-1 usually occurs. Furthermore, this finding revealed a latent stage of the virus, as recently observed in adults (Dundon et al. 2011; Dégremont et al. 2013). With regard to the mortality events, no mortality was observed throughout the experiment when a HP batch was grown alone in a tank or in a pond (Figs 4a and 5a), suggesting that HP spat could be free of OsHV-1 and subsequently mortality-free if grown in a safe environment. However, such culture areas are limited due to the range expansion of the virus throughout Europe, the USA, New Zealand and Australia (EFSA 2010; Cameron and Crane, 2011; Garcia et al. 2011; Burge and Friedman 2012; Jenkins et al. 2013; Paul-Pont et al. 2013). This, the feasibility of this approach is questionable. Moreover, the

Marennes-Oléron Bay is characterized by several thousand ponds, which could be used for this purpose. In contrast, WC batches always exhibited mortality, even when they were grown alone in a tank or in a pond. Additionally, both origins exhibited mortality when a WC batch and HP batches were grown together, although this phenomenon was only observed for one of the three tanks in the laboratory (Figs. 4b and 5b). The small number of WC oysters used for this condition combined with the low percentage of OsHV-1 infected animals at reception of the batch could explain this result. Together, our results suggested that OsHV-1 was horizontally transmitted from the WC batch to the HP batches in the mixed conditions in the laboratory and pond, and this transmission occurred quickly because the mortality began at nearly the same time in the various groups tested. This report is the first of such a transmission from naturally infected wild-caught spat to naïve hatchery-produced spat. Our study provides new information on the spread of the disease; to date, the horizontal transmission of OsHV-1 has only been demonstrated from unselected and asymptomatic adults to naïve spat (Dégremont et al. 2013), and all other studies have described the transmission of a homogenate prepared from infected oysters to larvae (Le Deuff et al. 1994; Burge and Friedman 2012) or between healthy and experimentally infected oysters under cohabitation conditions (Schikorski et al. 2011).

The mortality was higher for the WC1 batch at the mixed condition than for the separated conditions in the pond and the laboratory, as well as for all batches in the mixed condition in the pond and the laboratory in comparison to the field (Table 2). This finding confirmed that the mortality related to OsHV-1 is more likely and intense in confinement than in an open environment, as demonstrated by Garcia et al. (2011). Thus, the concentration of OsHV-1 particles could have been higher due to the small volume of the tank or the pond combined with

346 the flow-through system, exceeding the threshold of resistance for some oysters in this condition
347 as shown by Dégremont et al. (2013).

348 OsHV-1 was detected in both WC batches from April 2010. Similar reports have been
349 presented for numerous wild-caught batches (SMEL 2012). This finding suggests that the WC
350 batches were infected with OsHV-1 either in Marennes-Oléron Bay or Arcachon Bay during the
351 summer and fall of 2009. In both sites, the spawning season occurred during the summer 2009,
352 and the resulting larvae and spat could have been exposed to OsHV-1 particles and might have
353 been infected. Depending on the intensity of the viral challenge, a part of the cohorts might have
354 died due to OsHV-1 during the summer and fall of 2009, when the seawater temperature
355 exceeded 16°C. Another group may have survived and remained asymptomatic carriers of the
356 virus, and a last group might not have been in contact with the virus. Furthermore, our results
357 showed a higher mortality for the hatchery-produced batches than the wild-caught batches in the
358 field, as well as for the mixed condition in the pond and laboratory (Table 2). Even if a strong
359 genetic basis for mortality in juvenile *C. gigas* exists and the selected oysters were also resistant
360 to OsHV-1 (Dégremont et al. 2010a; Dégremont 2011), oysters of the WC batches are more
361 likely to be survivors of the primary infection in 2009. Thus, the WC and HP batches would
362 likely have exhibited comparable cumulative mortalities. To strengthen this hypothesis,
363 Dégremont et al. (2010b) showed that the survival rate of survivors was higher than in oysters
364 protected from the mortality risk factors.

365 The use of specific pathogen-free animals, such as the three HP batches for which OsHV-
366 1 was not detected at the beginning of the experiment, was advantageous over the use of wild-
367 caught spat for growth in an area where the pathogen has not yet been introduced. Nevertheless,
368 the utilization of such animals in areas where the disease is present usually leads to massive

mortality, such as that reported in the present study, but also in other oyster species, as demonstrated for *C. virginica* with *Perkinsus marinus* and *C. ariakensis* with *Bonamia ostreae* (Albright et al. 2007; Carnegie et al. 2008). Additionally, naïve WC spat would also benefit from an OsHV-1-free environment; not just naïve HP spat, but all WC spat should be considered as infected with OsHV-1, because the disease was detected in all areas where these animals are caught in the wild in France. Extreme care should then be taken with these animals to prevent the introduction of OsHV-1 into national and international pathogen-free areas, such as hatcheries, nurseries, ponds, or open sites.

Moreover, management strategies exist to limit the spread of the disease, its intensity, and the reservoir host. The mortality could be significantly reduced via selective breeding to improve the OsHV-1 resistance, decrease the oyster mortality, reduce the reservoir host, and limit horizontal transmission (Dégremont et al. 2010a; Dégremont 2011; Dégremont et al. 2013). This approach can be easily implemented for hatchery production, as it is now broadly used by some French commercial hatcheries. Establishing a control strategy for wild-caught spat remains more difficult because the genetic background includes cultured and wild diploid oyster populations. The development of a restoration program is one possibility that could help to introduce genetic resistance in these populations via the production of numerous disease-resistant strains in hatcheries, which would then be introduced in the field. Of course, such approaches require particular considerations, such as (1) a massive introduction of disease-resistant oysters in comparison to wild and cultured stocks at least several years in a row, (2) massive mortality due to OsHV-1 each year to obtain a constant selective pressure on the oyster stocks, (3) the significant reduction of the gene flow through the transfer of unselected oysters into the environment and (4) the identification of the spatial and temporal variability of disease refuges,

which would undeniably impact the development of resistance, as demonstrated in *C. virginica* for MSX and Dermo (Ford et al. 2012). If these issues are not addressed, restoration programs will have a limited impact, as demonstrated for *C. virginica* in the Yeocomico River (Carlsson et al. 2008).

CONCLUSIONS

The results of our study indicated that French wild-caught spat are infected with OsHV-1 and remain asymptomatic carriers until the environmental conditions favor the disease. The use of such oysters allows the spread of the disease in disease-free areas or disease-free animals, especially for unselected oysters. Unselected hatchery-produced spat could be protected from the mortality due to OsHV-1 when they are grown separate from infected stock, and disease-free water is used. Indeed, not using UV or using water pumped during a major mortality event in the field could contaminate the HP oysters with OsHV-1. Importantly, the HP oysters were always protected from the mortality risk factor in the present study, and care must be taken when oyster farmers use such animals, as they may have been grown in an area where OsHV-1 is present.

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, in particular Christophe Ledu, Max Nourry and Tanguy Guyader. This work was

412 funded by the Contrat de Projet Etat Région Poitou-Charentes, 2007 – 2013, Convention
413 n008/RPC-A-29 du 19 mai 2008.

414

415 LITERATURE CITED

416 Albright BW, Abbe GR, McCollough CB, Barker LS, Dungan CF (2007) Growth and mortality
417 of dermo-disease-free juvenile oysters (*Crassostrea virginica*) at three salinity regimes in
418 an enzootic area of Chesapeake Bay. J Shellfish Res 26:451-463

419 Arzul I, Renault T, Thebault A, Gerard A (2002) Detection of oyster herpesvirus DNA and
420 proteins in asymptomatic *Crassostrea gigas* adults. Virus Res 84:151-160

421 Burge CA, Friedman CS (2012) Quantifying Ostreid Herpesvirus (OsHV-1) Genome copies and
422 expression during transmission. Microb Ecol 63:596-604

423 Cameron A, Crane M (2011) Final Report: OsHV-1 μVar International Workshop, Cairns,
424 Queensland, Australia 9-10 July 2011. AusVet Animal Health Services and Fisheries
425 Research and Development Corporation, Canberra.

426 Carlsson J, Carnegie RB, Cordes JF, Hare MP, Leggett AT, Reece KS (2008) Evaluating
427 recruitment contribution of a selectively bred aquaculture line of the oyster, *Crassostrea*
428 *virginica* used in restoration efforts. J Shellfish Res 27:1117-1124

429 Carnegie RB, Stokes NA, Audemard C, Bishop MJ, Wilbur AE, Alphin TD, Posey MH, Peterson
430 CH, Burreson EM (2008) Strong seasonality of *Bonamia sp* infection and induced
431 *Crassostrea ariakensis* mortality in Bogue and Masonboro Sounds, North Carolina, USA.
432 J Invertebr Pathol 98:335-343

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

435 Dégremont L (2013) Size and genotype affect resistance to mortality caused by OsHV-1 in
436 *Crassostrea gigas*. Aquaculture 416-417:129-134

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

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

443 Dégremont L, Guyader T, Tourbiez D, Pépin JF (2013) Is horizontal transmission of the Ostreid
444 herpervirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival
445 status in adults to juveniles. Aquaculture 408-409:51-57

446 Dégremont L, Soletchnick P, Boudry P (2010c) Summer mortality of selected juvenile Pacific
447 oyster *Crassostrea gigas* under laboratory conditions and comparison with field
448 performance. J Shellfish Res 29:847-856

449 Dundon WG, Arzul I, Omnes E, Robert M, Magnabosco C, Zambon M, Gennari L, Toffan A,
450 Terregino C, Capua I, Arcangeli G (2011) Detection of Type 1 Ostreid Herpes variant
451 (OsHV-1 μvar) with no associated mortality in French-origin Pacific cupped oyster
452 *Crassostrea gigas* farmed in Italy. Aquaculture 314:49-52

453 EFSA (2010). Scientific Opinion on the increased mortality events in Pacific oysters, *Crassostrea*
454 *gigas*. EFSA J 8:1894-1953

455 Ford S, Scarpa E, Bushek D (2012) Spatial and temporal variability of disease refuges in an
456 estuary: Implications for the development of resistance. J Mar Res 70:253–277

457 Garcia C, Thébault A, Dégremont L, Arzul I, Miossec L, Robert M, Chollet B, Francois C, Joly
458 J-P, Ferrand S, Kerdudou N, Renault T (2011) Ostreid herpesvirus 1 detection and
459 relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Vet
460 Res 42:73

461 Goulletquer P, Le Moine O (2002) Shellfish farming and coastal zone management (CZM)
462 development in the Marennes-Oleron Bay and Charentais Sounds (Charente Maritime,
463 France): A review of recent developments. Aquacult Int 10:507-525

464 Guichard B, François C, Joly JP, Garcia C, Saulnier D, Pépin JF, Arzul I, Ommes E, Tourbiez D,
465 Faury N, Haffner P, Chollet B, Robert M, Renault T (2011) Bilan 2010 du réseau
466 REPAMO. Rapport IFREMER 22p

467 Jenkins C, Hick P, Gabor M, Spiers Z, Fell S, Gu X, Read A, Go J, Dove M, O'Connor W,
468 Kirkland P, Frances J (2013) Identification and characterisation of an ostreid herpesvirus-
469 1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis
470 Aquat Organ 105:109-126

471 Lacoste A, Jalabert F, Malham S, Cueff A, Gelebart F, Cordevant C, Lange M (2001) A *Vibrio*
472 *splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea*
473 *gigas* in the Bay of Morlaix (North Brittany, France). Dis Aquat Organ 46:139-145.

Le Roux F, Gay M, Lambert C, Waechter M, Poubalanne S, Chollet B, Nicolas JL, Berthe F (2002) Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. Aquat Living Resour 15:251-258.

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*. Bull Eur Ass Fish Pathol 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*. Dis Aquat Organ 24:149-157.

Littell RC, Stroup WW, Freund RJ (2002) SAS® for Linear Models. 4th ed. Cary, NC:SAS Institute Inc

Lynch SA, Carlsson J, Reilly AO, Cotter E, Culloty S.C (2012) A previously undescribed ostreid herpes virus 1 (OsHV-1) genotype detected in the pacific oyster, *Crassostrea gigas*, in Ireland. Parasitol 139:1526-1532

Martenot C, Fourour S, Oden E, Jouaux A, Travaille E, Malas JP, Houssin M (2012) Detection of the OsHV-1 µVar in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus 1 (OsHV-1). Aquaculture 338:293-296

Oden E, Martenot C, Berthaux M, Travaille E, Malas JP, 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

Paul-Pont I, Dhand N, Whittington R (2013) Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture 412-413:202-214

Peeler EJ, Allan Reese R, Cheslett DL, Geoghegan F, Power A, Thrush MA (2012) Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 µVar in the Republic of Ireland in 2009. Prev Vet Med 105:136-143

Pépin JF, Riou A, Renault T (2008) Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. J Virol Methods 149:269-276

Pernet F, Barret J, Le Gall P, Corporeau C, Dégremont L, Lagarde F, Pépin JF, Keck N (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquacult Env Interac 2:215-237

Petton B, Pernet F, Robert R, Boudry P (2013) Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas.* Aquacult Env Interac 3:257-273

Renault T, Le Deuff RM, Chollet B, Cochennec N, Gerard A (2000) Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-cultured spat *Crassostrea gigas* and *Ostrea edulis*. Dis Aquat Organ 42:173-183

Rohfritsch A, Bierne N, Boudry P, Heurtebise S, Cornette F, Lapègue S (2013) Population genomics shed light on the demographic and adaptive histories of European invasion in the Pacific oyster, *Crassostrea gigas.* Evol Appl 6:1064-1078

515 Roque A, Carrasco N, Andree KB, Lacuesta B, Elandaloussi L, Gairin I, Rodgers CJ, Furones
516 MD (2012) First report of OsHV-1 microvar in Pacific oyster (*Crassostrea gigas*)
517 cultured in Spain. Aquaculture 324:303-306

518 Sauvage C, Pépin JF, Lapègue S, Boudry P, Renault T (2009) Ostreid herpes virus 1 infection in
519 families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak:
520 Differences in viral DNA detection and quantification using real-time PCR. Virus Res
521 142:181-187

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

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

528 SMEL (2012) Suivi Sentinelle Interrégional. Bulletin n° 5. Syndicat Mixte pour l'Equipement du
529 Littoral. http://www.smel.fr/iso_album/bulletin_5_interegional.pdf

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

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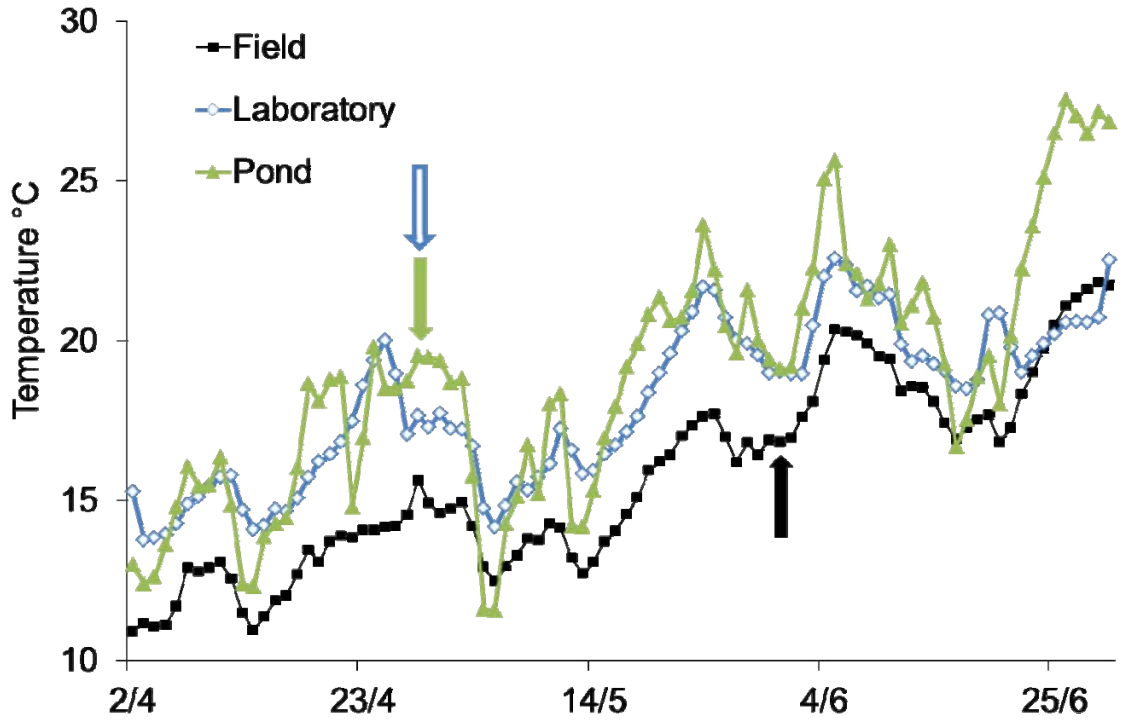
533

534 Fig. 1. Location of the three sites (F field, P ponds, L laboratory) on the Seudre River, Marennes-
535 Oléron Bay, France

536



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538 Fig. 2. Seawater temperature (°C) at the three sites throughout the experiment. The arrows

539 indicate the onset of a significant mortality event.

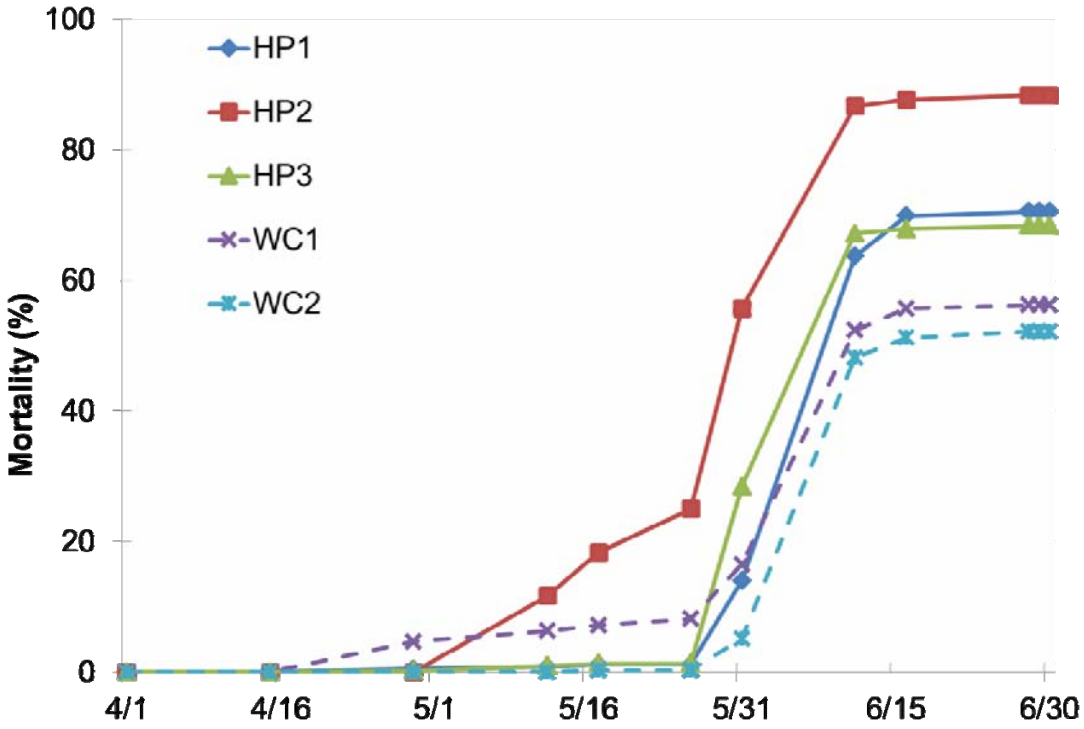

Fig. 3. Kinetics of the mortality for the three hatchery-produced batches (HP1 to HP3, solid lines) and the two wild-caught batches (WC1 and WC2, dashed lines) in the field from April to June 2010.

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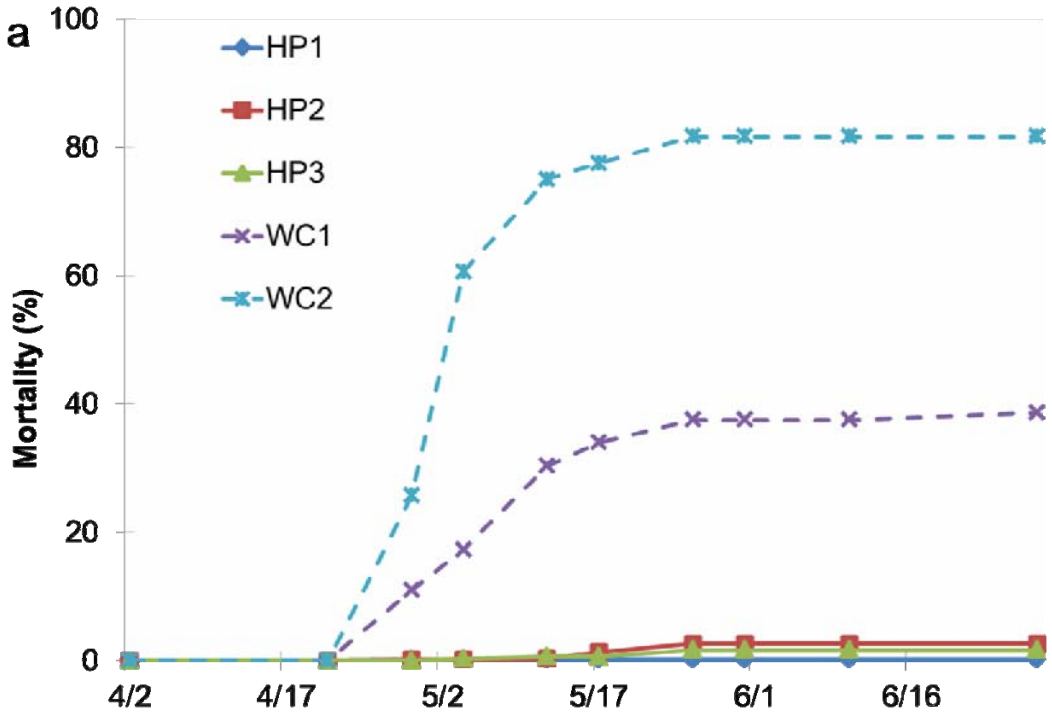
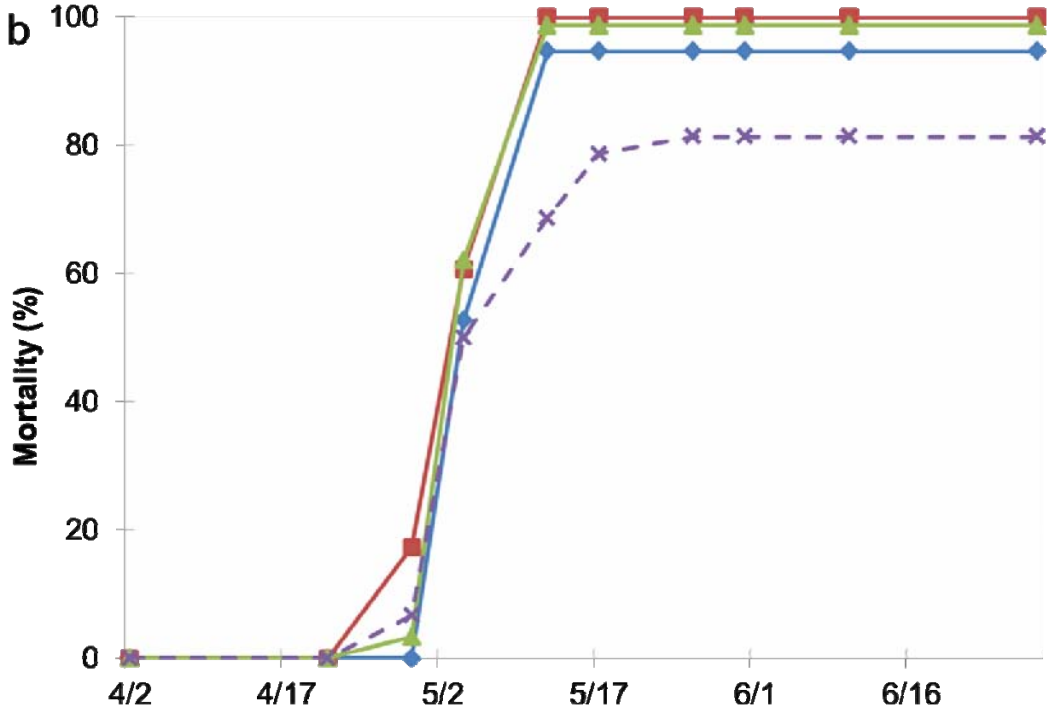

a
100
80
60
40
20
0
Mortality (%)
HP1
HP2
HP3
WC1
WC2
4/2
4/17
5/2
5/17
6/1
6/16

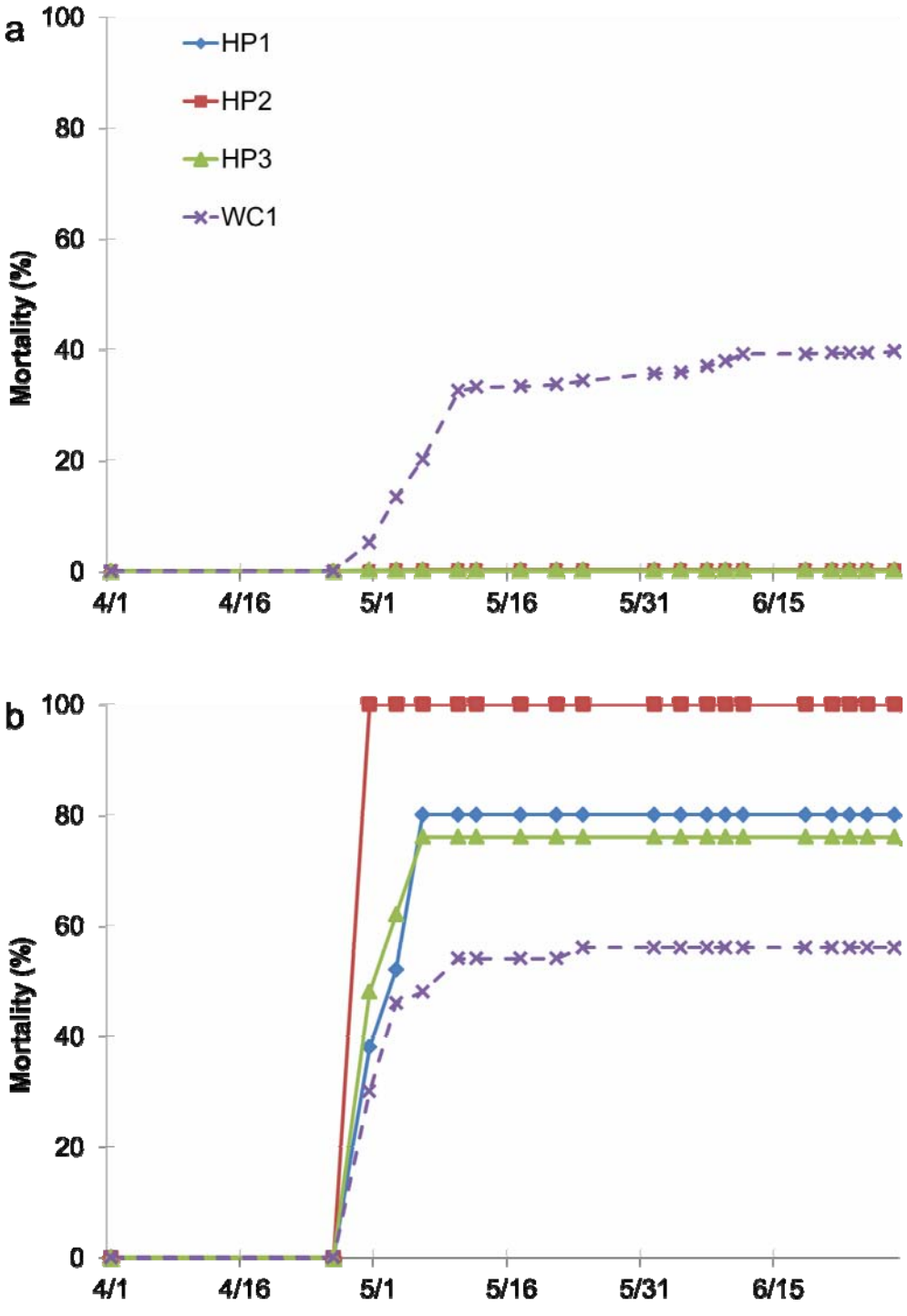
546


b
100
80
60
40
20
0
Mortality (%)
4/2
4/17
5/2
5/17
6/1
6/16

547

548 Fig. 4. Kinetics of the mortality for the three hatchery-produced batches (HP1 to HP3, solid lines)
549 and the two wild-caught batches (WC1 and WC2, dashed lines) in the pond from April to June
550 2010 for the separated (a) and mixed conditions (b).

551



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554 Fig. 5. Kinetics of the mortality for the three hatchery-produced batches (HP1 to HP3, solid lines)

555 and the wild-caught batch WC1 (dashed line) in the laboratory from April to June 2010 for the

556 separated (a) and mixed conditions (b). For the latter, the graph only shows the kinetics of the
557 mortality in the tank where mortality was observed.

558 Table 1. Summary of the batches and conditions tested

Site	Condition[a]	Batch tested	Number of replicates per batch	Number of oysters per replicate
Field[b]	Mixed	HP1, HP2, HP3, WC1, WC2	3 bags	200
Pond	Separated	HP1, HP2, HP3, WC1, WC2	3 bags	200
	Mixed	HP1, HP2, HP3, WC1	3 bags	50
Laboratory	Separated	HP1, HP2, HP3, WC1	3 tanks	200
	Mixed	HP1, HP2, HP3, WC1	3 tanks	50

559 [a] "Separated" indicates that only one batch was placed in the pond or tank, *i.e.* within-batch
560 cohabitation trial, while "mixed" means that all batches were placed in the pond or tank, *i.e.*
561 between-batches cohabitation trials.

562 [b] the separated condition was not tested because the batches could not be physically separated in

563 the field condition.

Table 2. Cumulative mortality (%) (mean and standard deviation among the replicates) of each batch in each condition and each site on June 30th

Condition	Batch	Laboratory[a]	Pond	Field
Mixed	HP1	27 ± 46	95 ± 9	71 ± 4
	HP2	33 ± 26	100 ± 0	88 ± 9
	HP3	26 ± 43	98 ± 2	68 ± 23
	WC1	19 ± 32	81 ± 16	56 ± 4
	WC2			52 ± 7
Separated	HP1	0 ± 0	1 ± 1	
	HP2	1 ± 1	3 ± 2	
	HP3	1 ± 1	2 ± 1	
	WC1	40 ± 2	39 ± 40	
	WC2		82 ± 1	

[a] To obtain the mortality rates for the tank representing the mixed condition for which a mortality outbreak was observed, the mortality rates should be multiplied by 3.

570 Table 3. Number of detected positive for OsHV-1 screening in living animals for each batch in
571 each condition and each site on June 30th

			Endpoint		
Condition	Batch	at deployment	Laboratory[a]	Pond	Field
Mixed	HP1	0/50	0/12-0/12	0/5	3/12
	HP2	0/50	0/12-ad	ad	4/12
	HP3	0/50	0/12-2/6	0/1	7/12
	WC1	7/50	0/12-1/12	0/12	1/12
	WC2	1/50			2/12
Separated	HP1		0/12	0/12	
	HP2		0/12	0/12	
	HP3		0/12	0/12	
	WC1		0/12	0/12	
	WC2			1/12	

572 [a] first number is given for the replicates without mortality, and the second numbers for the
573 replicate with mortality.

574 ad: all dead.