Synthesis of the “PLAN DE SAUVEGARDE” using selected all-triploid oysters to reduce the shortage of spat in France due to OsHV-1–associated mortality in Crassostrea gigas

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aSynthesis of the "PLAN DE SAUVEGARDE" using selected all-triploid oysters to reduce the shortage of spat in France due to OsHV-1–associated mortality in *Crassostrea gigas*

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ABSTRACT

Due to massive mortality of *Crassostrea gigas* spat in France since 2008, a "plan de sauvegarde" was set up from 2011 to 2014 (hereafter referred to as PS1 to PS4), in order to reduce the shortage of spat. This plan involved the participation of commercial hatcheries, the French Research Institute for Exploitation of the Sea (Ifremer), and the Direction des Pêches Maritimes et de l'Aquaculture (DPMA) of the French Ministry of Agriculture. It was based on selecting diploid lines of *C. gigas* for their higher resistance to the oyster herpesvirus OsHV-1 (2nR group), and one of these lines was subsequently tetraploidized (4nR group). Both the 2nR and 4nR groups were produced by Ifremer, and then transferred to commercial hatcheries that produced the selected triploids (3nR groups). We report here the mortality rates of the 3nR group for each of the four "plan de sauvegarde" campaigns and compare these with the mortalities of the classic production of commercial hatcheries (both 2n and 3n), benchmarks of selected (2nR group) and unselected (2n-control group) oysters produced by Ifremer, and wild-caught spat, representing a total of 104 diploid and triploids batches. For PS1, the 3nR group had a mean mortality of 67% and did not show any advantage over the 2n- and 3n-commercial groups, suggesting a lack of genetic progress in the 2nR and 4nR groups. For PS2, OsHV-1 resistance was increased in both the 2nR and 4nR groups and, consequently, the 3nR group exhibited a mean mortality of 52%, which was significantly lower than the mortality of the 2n- (87%) and 3n-(76%) commercial groups in 2012. Unfortunately, the mortality of the 3nR group reached 62% and 71% in PS3 and PS4, respectively, although it was expected to be lower than that in PS2. OsHV-1 DNA was quantified in the live oysters at deployment (1,356 oysters) and at the endpoint (1,171 oysters), as well as in moribund oysters sampled during peak mortality (539 oysters). The results strongly supported the involvement of this pathogen during the main mortality outbreak in May/June. Meanwhile, *Vibrio aestuarianus* was also suspected to cause unexpected mortality of PS3 oysters in August and

September, and it was detected in moribund PS4 oysters during both the mortality events, in May and July. Despite genetic improvement for OsHV-1 resistance, this translated into variable commercial genetic gain. This could be explained by the limited genetic backgrounds of the 2nR and 4nR groups, the reemergence of *V. aestuarianus* in France since 2012, the changing levels of genetic improvement in both the 3nR group and the commercial groups, as well as the limited broodstock genetic variation where small numbers of males were used. Results on growth and yield are discussed.

Keywords: triploids; mortality; *Crassostrea gigas;* OsHV-1; disease resistance

1. Introduction

French production of the Pacific cupped oyster *Crassostrea gigas* is based on both wild-caught and hatchery-produced spat, representing 70% and 30% of the cultivated spat, respectively. This proportion varies between years due to the amount of wild-caught spat, which strongly depends on diseases and environmental conditions as well as the number of collectors deployed by oyster farmers to harvest the spat.

Since 2008, massive mortality of spat has afflicted *C. gigas* in France. In the French context, since the first investigations of oyster herpesvirus (OsHV-1)-related mortality, both wild-caught and hatchery-produced seed, and both diploids and triploids, have been affected, with mortality rates usually exceeding 80% (Jenkins et al., 2013). Similar mortality has also been reported in other European countries, Morocco, Australia, New Zealand, and on the western coast of the USA during the same period (Burge and Friedman, 2012; Cameron and Crane, 2011; EFSA, 2010; Jenkins et al., 2013; Lynch et al., 2012; Martenot et al., 2012; Paul-Pont et al., 2013; Peeler et al., 2012; Roque et al., 2012). Disease investigations have revealed the involvement of OsHV-1. A particular OsHV-1 genotype (µvar) has been identified as the most aggressive causal agent associated with spat mortality since 2008 (Segarra et al., 2010) and was also found during a period of *C. gigas* mortality in 2004-2005 in Normandy (Martenot et al., 2012). Although OsHV-1 was suspected to be the principal causal agent of the mortality, it is noteworthy that this virus has been co-detected with different species of *Vibrio*, including *Vibrio* belonging to the *V. splendidus* clade and *V. aestuarianus* species, during several mortality events in France (Francois et al., 2009; Lemire et al., 2015; Saulnier et al., 2010) and New Zealand (Keeling et al., 2014). Additionally, mortality was reported to be higher in the presence of OsHV-1 associated with *Vibrio* species than in the presence of

74 OsHV-1 alone, indicating that the herpes virus appears neither essential nor sufficient to cause
75 juvenile deaths (Petton et al., 2015b).
76 In reaction to the mortality outbreaks that caused dramatic annual losses of juvenile oysters,
77 two complementary strategies were adopted in France. The first was that oyster farmers
78 increased their spat collection capacity tremendously. The second was that during the same
79 period the amount of hatchery-produced spat increased regularly each year, reaching
80 approximately three billion units in 2012 (Dégremont et al., 2016). This increase of hatchery-
81 produced spat was primarily driven by triploid oysters, which show better growth and yield
82 than their diploid counterparts (Hand et al., 2004). In addition, triploids are preferred over
83 diploids in the summer because diploids are less marketable when in spawning condition
84 (Allen and Downing, 1986; Nell, 2002). However, as these wild-caught and hatchery spat
85 were not intentionally bred for their resistance or tolerance against OsHV-1, these two
86 strategies ultimately appeared to be unproductive and cumulative mortalities remained very
87 high. A considerable number of the unselected spat was cultivated in field conditions, where
88 they could easily be infected with OsHV-1 when grown in a contaminated area and were then
89 capable of spreading the pathogens.
90 During the same period, breeding investigations revealed a high genetic basis for survival
91 during summer mortality events in juvenile *C. gigas* (Dégremont et al., 2010). It was also
92 shown that selected diploid oysters resistant to the summer mortality events were also
93 resistant to OsHV-1 in the context of the widespread and more severe mortality outbreaks in
94 France since 2008 (Dégremont, 2011). Additionally, the preliminary results of one previous
95 study showed that similar mortality rates were obtained in chemically induced triploids and
96 their corresponding diploids (Dégremont et al., 2016). This suggests that innate resistance to
97 OsHV-1 is not substantially altered by triploidization and that progress in the selective
98 breeding of diploid oysters for OsHV-1 resistance can be transferred to improve survival in

triploids. The most efficient method of producing triploid oysters is to breed tetraploids with diploids in a hatchery (Guo et al., 1996). This method is used in France where triploid spat are obtained after crossing diploid female stocks from private hatcheries with a stock of tetraploid males produced and maintained at the Ifremer hatchery in La Tremblade. Consequently, in order to provide commercial hatcheries with the best breeders for the production of genetically improved triploids, improved tetraploid lines were directly induced from the available selected diploid broodstocks using the method described by Benabdelmouna and Ledu (2015). In addition to the expected advantages introduced by selective breeding, the major expression of triploidy in oysters is the interruption of normal reproductive activity, rendering them functionally sterile (Guo and Allen, 1994). Triploid sterility is often mentioned as a very effective tool for the protection of native genetic resources from aquaculture escapees and to ensure the genetic confinement of transgenic organisms (Benfey et al., 1986; Thorgaard and Allen Jr, 1986).

Accordingly, there was general agreement in France to use selected triploids in the frame of a collaborative preservation plan named the “plan de sauvegarde” that was developed to compensate for the annual spat losses. In this temporary plan, under the supervision of national authorities and oyster farmer representatives, Ifremer and all the French commercial hatcheries producing triploids worked in collaboration in order to produce a sufficient amount of improved all-triploid spat that could be used by oyster farmers.

Practically, the induction method of tetraploidy was used to generate improved tetraploid broodstock (4nR) *de novo* from elite diploid lines (2nR) obtained through mass and/or family-based selection. After that, crossing both 4nR males and 2nR females was accomplished in the commercial hatcheries in order to mass produce genetically improved all-triploids (3nR), which were then sold to oyster farmers. This approach was implemented in France for 4 years, until 2014, and the present manuscript describes the results obtained during this period.

2. Materials and methods

2.1. Schedule of the "plan de sauvegarde."

Four "plan de sauvegarde" campaigns, hereafter called PS1 to PS4, were followed from 2011 to 2014, one PS campaign per year. The time frame was the same for the four PSs. One or two years earlier (Year n-2 and n-1), unselected (control) or selected (R) diploid (2n) and tetraploid (4n) broodstocks were produced by the Laboratory of Genetics and Pathology of Marine Molluscs (LGPMM), at the Ifremer hatchery in La Tremblade. Selected diploid oysters (2nR) were provided by the LGPMM to the commercial hatcheries to optimize the conditioning of a large volume of oysters in Year n-1. Then, the LGPMM provided selected tetraploid male oysters (4nR) to the commercial hatcheries in Year n so that they could produce the selected triploid oysters (3nR) by mating 2nR females with 4nR males. In order to evaluate the resistance of the 3nR oysters, the commercial hatcheries produced commercial batches of diploid (2n-commercial) and triploid (3n-commercial) oysters, using their own 2n stocks and unselected 4n stocks produced and provided by the LGPMM. In addition, the LGPMM produced unselected (2n-control) and selected (2nR) diploid oysters as benchmarks. For PS1 and PS3, wild oysters (2n-wild) caught in Year n were also used. At the end of Year n, the commercial hatcheries send 1000 spat per batch of all the 3nR batches produced to LGPMM, at a size of T6 (retained on a 6 mm mesh size sieve), which is the size most often sold to oyster farmers in France. They also sent the LGPMM some 2n-commercial and 3n-commercial batches for the survey. Finally, the LGPMM was responsible for the evaluation of all the oyster groups (2n-wild, 2n-commercial, 3n-commercial, 3nR, 2nR, and 2n-control) in Year n+1. These evaluations were done in 2011, 2012, 2013, and 2014 for PS1, PS2, PS3, and PS4, respectively. To summarize, and taking the case of PS1 as an example, selected

broodstocks (2nR and 4nR) were produced in 2009. The oyster groups were produced or caught in 2010 and evaluated in 2011. The dates of production and the number of oyster batches within each group for each PS campaign are given in Table 1.

2.2. Biological material

2.2.1. Groups produced by the commercial hatcheries

Commercial hatcheries produced the 2n-commercial, 3n-commercial, and 3nR groups.

2.2.1.1. 2n-commercial group

The 2n-commercial group was produced from the 2n broodstocks owned and selected by the participating hatcheries. There is no information about the level of resistance to OsHV-1 of these stocks. However, it could be supposed that their level of resistance to OsHV-1 increased from PS1 to PS4 as hatcheries developed selective breeding programs to enhance resistance to OsHV-1, as demonstrated by Dégremont et al. (2015a) and Dégremont et al. (2015b). The number of batches used for this group ranged from two for PS4 to six for PS3 (Table 1).

2.2.1.2. 3n-commercial group

The 3n-commercial group was produced by mating 2n females of the broodstock owned by each participating hatchery with unselected 4n males provided by Ifremer. Usually, a cross involves several dozen or hundreds of 2n females and one to six 4n males.

The 4n males were from the same stock that was provided since 2009 to all commercial hatcheries for French all-triploid production. This stock was initially produced in 2007 by induction from unselected diploid parents using the “direct method” described by Benabdelmouna and Ledu (2015). For PS1 and PS2, tetraploid males from the second and third generation of this initial broodstock, respectively, were used. In order to use selected tetraploid progenitors during the following PS campaigns, the first and second generations of tetraploids for which a unique event of mass selection occurred to enhance their resistance to

175 OsHV-1 were used for PS3 and PS4, respectively. The number of batches used for this group
176 ranged from four for PS1 to seven for PS2 (Table 1).

177 2.2.1.3. 3nR group

178 The 3nR group was produced by crossing 2nR females and 4nR males, both selected and
179 provided by Ifremer. Usually, a cross involves several dozen or hundreds of 2nR females and
180 one to six 4nR males.

181 The 2nR and 4nR oysters were the progenies of families selected for their higher resistance to
182 summer mortality during the MOREST program (MORtalités ESTivales) (Dégremont et al.,
183 2010; Samain and McCombie, 2007). The founder populations of the 2nR and 4nR oysters
184 were based on two males and four females (Dégremont et al., 2010). For PS1 and PS2, the
185 2nR oysters were the fifth generation of seven families (R1 to R7 - year of production 2007),
186 and the sixth generation of two of them (R1 and R5 - year of production 2009), which were
187 subsequently identified as resistant to infection by OsHV-1 (Dégremont, 2011). In addition,
188 the seventh generation of the families R1 and R5 (year of production 2010) were also
189 provided to the commercial hatcheries for PS2. For PS3 and PS4, the 2nR oysters were the
190 eighth generation of the R1 and R5 families (years of production 2010 and 2011).

191 Each selected family was reproduced for five or six generations between 2002 and 2009,
192 without any further selection, by always using genitor oysters that had been protected from
193 the mortality occurring in the field. In contrast, the parents of the seventh and eighth
194 generations used survivors of mortality outbreaks related to OsHV-1 in field conditions,
195 meaning that they had one and two rounds of mass selection for survival, unlike the other
196 generations. More details on their production and the estimation of their survival against
197 OsHV-1 are provided in Dégremont (2011) and Dégremont (2013).

198 Selected tetraploid males (4nR) were gradually obtained from 2nR using the direct and
199 indirect induction methods described by Benabdelmouna and Ledu (2015). For PS1, tetraploid

males (4nR5) were directly induced using diploid oysters from the fifth generation of the R5 family, which showed relatively high survival at the spat stage in 2009. For PS2, the 4nR group used the selected tetraploid males (4nR55), which were obtained from an induced crossing of 4nR5 males with 2nR females from the seventh generation of the R5 family, which was identified as the best elite diploid family for OsHV-1 resistance. For PS3 and PS4, the 4nR oysters were successive generations of the 4nR55 stock.

The number of batches used for this group ranged from seven for PS4 to 11 for PS3 (Table 1).

2.2.2. Production by the LGPMM

2.2.2.1. 2n-control group

The 2n-control group used wild oysters sampled from the Marennes-Oléron Bay. Each cross was done by a mass spawning of thirty adults. The number of batches used for this group ranged from two for PS2/PS4 to five for PS1 (Table 1).

2.2.2.2. 2nR group

From PS1 to PS3, the 2nR group used siblings of the 2nR oysters provided to the commercial hatcheries to produce the 3nR groups. Similar to the 2n-control, each cross was done by a mass spawning of thirty adults. In addition, two other stocks of 2nR were produced for PS3, and only those were used for PS4. These two stocks were the third generation of mass selection for survival and resistance to OsHV-1 infection and further descriptions are given in Dégremont et al. (2015a). The number of batches used for this group ranged from two for PS4 to five for PS1 (Table 1).

2.2.3. Wild group

Wild-caught spat were tested in PS1 and PS3. For PS1, two origins were used, the Marennes-Oléron Bay and the Arcachon Bay, as these two bays account for approximately 80% of wild-

225 caught production. For PS3, the same two origins were used and a third origin, Bourgneuf
226 Bay, was added.
227
228 2.3. Experimental design
229
230 All groups were tested at two sites within the Marennes-Oléron Bay, where severe mortality
231 outbreaks related to OsHV-1 have been reported in *C. gigas* since 2009 (Dégremont et al.,
232 2015a). The sites were at Agnas (1°10'35" W, 45°52'14" N) and La Floride (1°09'15" W,
233 45°48'12" N).
234 Seawater temperature was recorded every hour throughout the study using two ThermoTrack
235 probes (Progesplus, 59780, Willems, France) at each site. The dates of deployment are
236 reported in Table 1. At each site and for each oyster batch, one bag containing 200 oysters
237 was attached to an iron rack. Mortality was recorded monthly from deployment to the
238 endpoint, which occurred in September or October (Table 1). At deployment and the
239 endpoint, the yield of each batch at each site was recorded by weighing the total weight of live
240 oysters and 30 to 50 oysters per batch per site were individually weighed, this number being
241 lower for batches with high mortality.
242
243 2.4. Detection and quantification of OsHV-1 DNA and *Vibrio aestuarianus* DNA
244
245 On reception at Ifremer, live oysters from each group were sampled for the detection and
246 quantification of OsHV-1. Similarly, live oysters were also sampled at the endpoint, while
247 moribund oysters were sampled during peak mortality in May/June. This was done for each
248 PS, corresponding to a total of 1336 live oysters on reception, 500 moribund oysters during
249 the first peak of mortality, and 1171 oysters at the endpoint. As survival was checked once a

month at each site, moribund oysters with tissue acceptable for DNA extraction were sometimes scarce, explaining the lower number of oysters sampled during mortality events. The number of samples analyzed for the detection and quantification of OsHV-1 for each PS campaign is indicated in Table 2, while that for each group within each PS campaign is indicated in Supplementary Table 1. In addition, 39 moribund oysters from PS4 were also sampled in July for OsHV-1 quantification. Finally, quantification of *V. aestuarianus* was also performed on all moribund oysters sampled in PS4. Tissues (mantle + gills) were stored in ethanol at -20°C until disease analysis.

Total DNA was extracted from the tissue fragments using the QIAgen (Hilden, Germany) QIAamp tissue mini kit combined with the QIAcube automated sample preparation system, according to the manufacturer's protocol. The amount of total DNA was adjusted to 5 ng/µl following NanoDrop (Thermo Scientific, Waltham, USA) measurement. A real-time PCR assay was conducted using MX3000 and MX3005 Thermocyclers (Agilent, Santa Clara, USA) and the Brilliant III Ultrafast kit (Stratagene). Each reaction was run in duplicate in a final volume of 20 µl containing a DNA sample (5 µl at 5 ng/µl), 200 nM of each primer (for OsHV-1: DPF 50 ATT GAT GATGTG GAT AAT CTG TG 30 and DPR 50 GGT AAA TAC CAT TGG TCT TGTTCC 30 (Webb et al., 2007); for *V. aestuarianus*: DNAj-F 50 GTATGAAATTTTAACTGACCCACAA30; and DNAj-R 50 CAATTTCTTTCGAACAACCAC30 (Saulnier et al., 2009)), and 200 nM of oligonucleotide probe (for *V. aestuarianus* DNAj probe 50 TGGTAGCGCAGACTTCGGCGAC). The real-time PCR cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 20 s. The standard curve was determined using serially diluted titrated plasmids provided by the National Reference Laboratory (Ifremer La Tremblade). For OsHV-1 DNA quantification, melting curves were also plotted (55–95°C) to ensure that a single PCR product was amplified for each set of primers. Negative controls

(without DNA) were included.

2.5. Data analyses

All statistical analyses were conducted using SAS® 9.4 software.

2.5.1. Mortality

Mortality at the endpoint was analyzed within and among PS campaigns with a binomial logistic regression equation using the GLIMMIX procedure. Site, defined as a fixed effect, and age and size at deployment, defined as covariates, were all found to be nonsignificant and were subsequently dropped from the analysis. The following model was run:

$$Logit\ (Y_{ijk}) = \mu + group_i + batch(group)_j$$

where Y_{ijk} is the probability that the k*th* oyster from the *j*th batch of the *i*th group (2n-wild, 2n-commercial, 3n-commercial, 3nR, 2nR, or 2n-control) will die, and μ is the intercept; $group_i$ is a fixed effect, $batch(group)_j$ is a random effect.

Pairwise comparisons between the 3nR group and the other groups were conducted and the corresponding odds ratios and their 95% confidence intervals presented.

2.5.2. Growth and daily yield

For each PS campaign, the individual weight data were log transformed and analyzed using the MIXED procedure by running an ANCOVA with time as a covariate.

$$\text{Log}(Y_{ijk}) = \mu + time + group_i + batch_{j(i)} + time*group_i + time*batch_{j(i)} + \varepsilon_{ijk}$$

where μ is the intercept, *time* is the covariable, $group_i$ is a fixed effect (2n-wild, 2n-commercial, 3n-commercial, 3nR, 2nR, or 2n-control), batch is a random effect and ε_{ijk} is the

297 error term. The slopes of the relationships between the covariate (time) and either group or
298 batch represent direct measures of growth.
299 The daily yield was defined as follows:
300 Daily yield = 100 x (TWF-TWI) / (TWI x time)
301 Where TWI and TWF are the log transformed initial and final total weights of all live oysters,
302 and time is the duration in days between deployment and the endpoint.
303 The same model used for growth was used for the daily yield, except that the time and
304 interactions were not included.
305

306 **3. Results**

307

308 3.1.1. Seawater temperature

309 From August 2010 to December 2014, the monthly seawater temperature ranged from 5.8°C
310 in February 2012 to 21.4°C in August 2012 (Figure 1). In France, mortality related to OsHV-1
311 is usually reported at sea temperatures near and above 16°C, which corresponds to the period
312 from May to September.

313

314 3.1. Mortality

315 Low mortality (<20%) was observed during September 2010 and September 2013 in PS1 and
316 PS4, respectively (Figures 1 & 2). For all PS campaigns, the main mortality outbreak occurred
317 in May/June, and then mortality generally decreased until the endpoint, except for in PS3
318 when a second mortality event occurred between August and September 2013 (Figures 1 &
319 2). At the endpoint, the mean final mortality was not significantly different between sites and
320 reached 68.5 ± 0.5%, 58.1 ± 4.4%, 61.1 ± 1.8%, and 58.1 ± 2.1% in PS1 to PS4, respectively
321 (Figures 1 & 2).

For all PS campaigns, the 2nR group had the lowest mortality, ranging from 22.5% to 40%, while the 2n-control group had the highest, ranging from 82.5% to 96.7%, except for PS2 which had a 2n-control group mortality of only 58.9% (Table 3) (Figure 3). When the 2n-wild group was tested, the mean mortality was 73.5% and 73.0% in PS1 and PS3, respectively. For the 2n- and 3n-commercial groups, mortality was higher in PS1 and PS2 (64.1 to 86.8%) than it was in PS3 and PS4 (53.0 to 58.4%) (Table 3) (Figure 3). The mean mortality of the 3nR group was 67.3% for PS1, 51.6% for PS2, 61.5% for PS3 and 70.3% for PS4 (Table 3) (Figure 3).

Significant differences in mortality at the endpoint were reported among groups for each PS campaign ($P<0.0001$ from PS1 to PS3; $P=0.0113$ for PS4). For PS1, 3nR oysters had significantly lower mortality than the 2n-control group, with the odds ratio of death in 2n-control oysters 15.6 times that of the 3nR oysters. They also showed significantly higher mortality than the 2nR-control group with the odds ratio of death in 2nR oysters 0.3 times that of 3nR oysters (Table 4). Thus, the 3nR, 2n-, and 3n-commercial groups showed no significant differences in mortality at the endpoint in PS1 (Table 4). Similar results were obtained for PS3 and PS4, except that the 3nR and 2n-control oysters showed no significant differences in mortality in PS4 (Table 4). For PS2, the 3nR oysters showed significantly lower mortality than the 2n and 3n-commercial groups with the odds ratio of death in the 2n and 3n-commercial oysters 6.8 times and 3.9 times that of the 3nR oysters, respectively. Meanwhile, 3nR oysters showed significantly higher mortality than the 2nR-control group with the odds ratio of death in 2nR oysters 0.3 times that of the 3nR oysters (Table 4).

Among all the PS campaigns, the 3nR oysters had significantly lower mortality than the 2n-control group, and significantly higher mortality than the 2nR-control group. The odds ratios of death in the 2n-control, 2n-wild, 2n-commercial, and 3n-commercial oysters were 5.3, 1.6, 1.5, and 1.2 times that of the 3nR oysters, respectively, whereas in the 2nR-control group it

347 was 0.3 times lower than that of the 3nR oysters (Table 4).

348

349 3.2. Quantification and detection of OsHV-1 and *V. aestuarianus*.

350 On reception, OsHV-1 was detected in 1 to 23% of the alive oysters analyzed, but the quantity
351 of OsHV-1 DNA was low from PS1 to PS3 ($<10^3$ copies per mg of fresh oyster tissue) (Table
352 2). In contrast, a much higher amount of OsHV-1 DNA was detected in the positive oysters
353 ($>10^6$ copies) in PS4, but on closer inspection, only two oysters displayed such values, while
354 the others contained less than 10^4 copies per mg.

355 During the mortality event in May/June, OsHV-1 DNA was detected in 89% and 100% of the
356 moribund oysters analyzed, ranging from 2×10^4 to 8×10^7 copies of OsHV-1 DNA per mg of
357 fresh oyster tissue (Table 2). Similar values were observed for the moribund oysters sampled
358 during July in PS4. Regarding the detection of *V. aestuarianus*, this pathogen was detected in
359 12% and 67% of the moribund oysters analyzed in May/June and July, respectively, ranging
360 from 1×10^7 to 3×10^6 copies of bacterial DNA per mg of fresh oyster tissue (Table 4). Co-
361 detection of OsHV-1 and *V. aestuarianus* was observed in 38 of the 155 moribund oysters
362 sampled in May/June 2014 and July 2014.

363 At the endpoint, the prevalence of OsHV-1 in live oysters was lower, ranging from 10% in
364 PS2 to 55% in PS4. The amount of OsHV-1 DNA was lower in PS1 and PS3 (with $<10^3$
365 copies of OsHV-1 DNA per mg of fresh oyster tissue) than it was in PS3 and PS4 (with $>10^5$
366 copies of OsHV-1 DNA per mg of fresh oyster tissue) (Table 2).

367

368 3.3. Growth and Yield.

369 At deployment, the mean individual weights among groups were 1.2 g in PS1 and PS2, 2.6 g
370 in PS3 and 0.4 g in PS4. At the endpoint, the mean individual weights were 9.9 g, 27.1 g, 22.3
371 g, and 25.2 g from PS1 to PS4, respectively. The box plots for the individual weights of each

372 group at deployment, and at the endpoint, are represented in Figure 4. Growth was not
373 significant among the groups for any of the PS campaigns with *P*=0.15 for PS1, *P*=0.17 for
374 PS2, *P*=0.09 for PS3, and *P*=0.79 for PS4. In contrast, batches nested within the groups
375 displayed significant differences in growth in each PS campaign (*P<0.0001*).
376 The daily yield was significantly different among groups in PS1 (*P<0.0001)* with the lowest
377 yield for the 2n-control group (-0.11% per day) while the other groups had similar yields
378 ranging from 0.14 % per day for the 2n-wild group to 0.24 % per day for the 3n-commercial
379 group (Figure 5). For PS2, the yield was significantly higher for the 3nR group (0.30 % per
380 day) in comparison to the other groups (*P*=0.0176) (Figure 5). For PS3, the yield was
381 significantly lower in the 2n-control group (0.04 % per day), while the 3n-commercial, 2n-
382 commercial, 2n-wild, and 3nR groups had intermediate yields (0.16 to 0.19% per day) and the
383 2nR group had the best yield (0.25 % per day) (*P<0.0001)* (Figure 5). Finally, the yield was
384 significantly different among the groups in PS4, with the lowest for the 2n-control group (0.18
385 % per day) and the highest for the 3n-commercial group (0.33% per day) (*P<0.0001)* (Figure
386 5).

387

388 **4. Discussion**

389

390 Due to the massive mortality of *C. gigas* spat related to OsHV-1, since 2008, the "plan de
391 sauvegarde" was set up with the main goal of reducing the shortage of spat in France from
392 2011 to 2014. In 2009, oysters selected for their higher resistance to summer mortality during
393 the MOREST program (MORTalités ESTivales) were also found to exhibit a higher resistance
394 to infection by OsHV-1 (Dégremont, 2011; Samain and McCombie, 2007). These oysters had
395 lower mortality than unselected ones but could experience high mortality (51-54%) when
396 faced with OsHV-1 at size smaller than 5 g (Dégremont, 2013), which was the case during
397 this study at sizes of around 1 g at deployment (Figure 4). For the "plan de sauvegarde", the
398 genetic background was limited to 2 to 7 families in the 2nR groups, and only one of them
399 (R5) was used to produce the 4nR group. Regardless of the performances of the 3nR group,
400 the duration of the "plan de sauvegarde" was defined until 2014 to allow time for commercial
401 hatcheries to develop their own selected oysters.

402

403 For each PS campaign, peak mortality occurred in May/June when seawater reached and
404 remained above 16°C (Figure 1). Such observations are common for OsHV-1-associated
405 mortality under field conditions in France (Dégremont, 2013; Dégremont et al., 2015a; Pernet
406 et al., 2012; Petton et al., 2015a). The detection of a large amount of OsHV-1 DNA in most of
407 the moribund oysters during the mortality outbreak in May/June strongly supports that OsHV-
408 1 was one of the main causes of mortality (Table 2). Interestingly, OsHV-1 and *V.*
409 *aestuarianus* DNA were both detected in moribund oysters from PS4 in July 2014 (Table 3).
410 Usually, the prevalence and quantity of OsHV-1 DNA in live oysters decrease markedly after
411 an OsHV-1-related mortality outbreak (Dégremont, 2011; Paul-Pont et al., 2013; Pernet et al.,
412 2012) and survivors are supposedly genetically resistant to subsequent OsHV-1 exposure. The

presence of a dual infection during and after the main peak of mortality in May/June suggests that the two pathogens could interact and kill the *C. gigas* spat that survived the primary infection with OsHV-1, as recently described for *C. gigas* juveniles in field conditions by Azéma et al. (2017a). To our knowledge, this finding is the second reported in the literature and requires further investigation.

Across the four PS campaigns, the 3nR group only showed significant decreases of mortality over the 2n- (-35%) and 3n-(-24%) commercial groups in PS2 (Table 3) (Figure 3) and even showed higher mortality than the commercial groups in PS3 and PS4. In contrast, the 2nR group performed consistently from PS1 to PS4, as did the 2n-wild group, and the 2n-control (Table 3) (Figure 3).

The first hypothesis which could explain these results relies on the level of selection to increase resistance to OsHV-1 infection in the parents of the 3nR group, as well as for those used to produce the 2n- and 3n- commercial groups. Indeed, the progress in selection for disease-resistant strains can be advanced through both the diploid and tetraploid lines as demonstrated in *Crassostrea virginica* (Dégremont et al., 2012). In our study, the level of selection of the parents for the 3nR group increased from PS1 to PS2 for both the 2nR and 4nR groups and also from PS2 to PS3/PS4 but only for the 2nR group. Consequently, this could explain the lower mortality of the 3nR oysters in PS2 (52%) than in PS1 (67%). Similarly, the lower mortality of the 3nR group (52%) over the 2n- and 3n-commercial groups in PS2 (76-87%) (Table 3) suggests that the genetic improvement for the resistance to OsHV-1 infection was higher for the 2nR and 4nR broodstocks than it was for the 2n and 4n broodstocks used by commercial hatcheries. While it was expected to observe a similar trend for the 3nR group in PS3 and PS4 as the 2nR broodstock went through an additional round of

438 selection compared to the 2nR broodstock in PS2, the 3nR group in PS3/PS4 had a higher
439 mortality than the 3nR group in PS2 (+10% and +19%, respectively) (Table 3). Although this
440 could be explained by the environment which varies temporally between the PS campaigns, it
441 is noteworthy that both the 2n- and 3n- commercial groups showed also lower mortality than
442 the 3nR group in PS3, suggesting a higher resistance from the 2n and 4n broodstocks used by
443 the commercial hatcheries than the 2nR and 4nR oysters used to produce the 3nR group.
444 Unfortunately, while the commercial tetraploid broodstock in PS3/PS4 was improved through
445 mass selection for OsHV-1 resistance, compared to the corresponding tetraploid broodstock in
446 PS1/PS2, no information is available from the commercial hatcheries regarding their own
447 selective breeding programs of diploid broodstocks. Nevertheless, the genetic gain of
448 resistance to OsHV-1 infection is significant over several generations of mass selection when
449 unselected stocks are used as the base population (Dégremont et al., 2015a). Also, high
450 variation exists among different spawns of 2n with 4n stocks due to a low effective population
451 size (restricted numbers of parents in the spawn), especially if the studied trait is highly
452 heritable (Azéma et al., 2017a; Azéma et al., 2017b). It would have been unlikely to obtain
453 the right combination between unrelated 2n and 4n stocks to obtain a higher resistance to
454 OsHV-1 infection in the 3n-commercial oysters than that in the 3nR oysters in PS3 and PS4.
455
456 The second hypothesis concerns the possible implications of another cause of mortality in PS3
457 and that the selection criterion based on resistance to OsHV-1 infection was inefficient in
458 responding to it. Interestingly, contrary to PS1 and PS2, a second mortality event was reported
459 in PS3, specifically between August and the endpoint in 2013 (Figure 1). Unfortunately no
460 moribund oysters were sampled during this period, but it was suggested that another pathogen
461 could have been implicated along with or without OsHV-1. This hypothesis was raised due to
462 mortality driven by the pathogenic bacteria *Vibrio aestuarianus* affecting market-sized adults

463 in France since 2012 (Azéma et al., 2015; Goudenège et al., 2015). It was also subsequently
464 found to affect all oyster stages (Azéma et al., 2016). The size of the oysters at the endpoint of
465 PS3 in September 2013 ranged from 14 to 32 g (Fig. 4) corresponding to the juvenile stage,
466 and indicating that they could be highly impacted by *V. aestuarianus*, as observed by Azéma
467 et al. (2017b). For mortality occurring from August to the endpoint, the 3nR group was the
468 most susceptible group (data not shown). Similarly, one of the batches of the 2nR group was
469 descended from a MOREST family and showed a much higher mortality (62%) than the other
470 two batches of the 2nR group (15% and 16%) (data not shown), as suggested by the box plot
471 of the 2nR group for PS3 (Figure 3). It could thus be supposed that the 2nR and 4nR groups
472 used to produce the 3nR group, which was based on a narrow founder population of two
473 males and four females selected during the MOREST program, could be particularly
474 susceptible to the causal agent of the mortality observed between August and September. In
475 contrast to PS3, disease sampling in PS4 detected *V. aestuarianus* at a low prevalence (12%)
476 during the peak mortality in May/June 2014. Nevertheless, the amount of bacterial DNA was
477 high and, more importantly, co-infection with OsHV-1 was reported for all 14 moribund
478 oysters (Table 2). The prevalence of *V. aestuarianus* increased to 67% in July, and 90% of the
479 oysters positive for *V. aestuarianus* were also positive for OsHV-1 (Table 2). Lately, variation
480 in susceptibility to OsHV-1 and *V. aestuarianus* was demonstrated among diploid stocks of *C.*
481 *gigas* (Azéma et al., 2017b) as well as between diploids and triploids (Azéma et al., 2016).
482 Furthermore, dual experimental infections with *V. aestuarianus* and OsHV-1 showed that
483 oysters experienced dramatic mortality rates, even those selected for higher resistance to
484 OsHV-1 (Azéma et al., 2016). The lack of genetic correlation between resistance to OsHV-1
485 infection and resistance to *V. aestuarianus* infection indicates that selection to improve
486 resistance to OsHV-1 infection should neither increase nor decrease resistance to *V.*
487 *aestuarianus* infection (Azéma et al., 2017b). Thus, it would be possible to select oyster

488 families with either dual resistance, dual susceptibility, or resistance to one of the diseases and
489 susceptiblity to the second disease (Azéma et al., 2017b). Unfortunately, the MOREST
490 families used to produce the 3nR group were based on a very limited genetic background (two
491 males and four females), and might have been highly susceptible to *V. aestuarianus*, losing
492 their advantage of OsHV-1 resistance when exposed to the bacteria. Our experience reveals
493 that selective breeding programs used in aquaculture must be thoroughly adapted to face
494 such events by using multiple germplasms/large populations of animals. Nevertheless, the
495 "plan de sauvegarde" was transient and ended in 2014. The emergence of a new, highly
496 virulent clonal strain of *V. aestuarianus* is unlikely (Goudenège et al., 2015), and it was
497 unfortunate that the re-emergence of *V. aestuarianus* occurred during the "plan de
498 sauvegarde".
499
500 Globally, the 2nR, 2n-control, and 2n-wild groups had consistent results across the four PS
501 campaigns (Figure 3). The 2n-control group, produced from wild broodstocks, had showed
502 mortality from OsHV-1, reaching 97% in PS1 (Table 3) (Figure 3). The high mortality of the
503 2n-control suggests the low frequency of occurrence of genetic resistance in the wild
504 populations of *C. gigas* sampled, and this result contrasted with the lower mortality of the 2n-
505 wild group in PS1 (74%). Nevertheless, while the life-history of the 2n-control is known from
506 spawning to inclusion in the survey, no such information is known for the 2n-wild group
507 before their reception for the survey. Thus, part of the population could already have been
508 exposed to OsHV-1 during their larval phase, recruitment, or growing phase before their
509 reception by Ifremer, and this mortality related to OsHV-1 would not have been included in
510 the final mortality rate recorded during the survey. This fact is also true for all groups
511 received from commercial hatcheries, although oysters might have remained unexposed to
512 OsHV-1 in the hatchery and in nursery inland growing facilities due to biosecurity systems.

513 Additionally, the lower mortality of the 2n-control group in PS2 compared to those in the
514 other PS campaigns could be explained by one of the 2n-control batches being older and
515 larger than the others produced across all PS campaigns, knowing that resistance to OsHV-1
516 increases with age and especially with size (Dégremont, 2013).
517 The 2n- and 3n-commercial groups had lower mortality than the 3nR group in PS3 and PS4
518 (Table 3), suggesting their higher resistance to mortality events. In addition, the mortality of
519 commercial oysters was lower in PS3 and PS4 (53 to 58%) compared to that in PS1 and PS2
520 (64 to 87%) (Table 3). As described above, these results could be explained by (1) the
521 breeding programs to enhance the OsHV-1 resistance of diploid and tetraploid broodstocks
522 used by the commercial hatcheries, (2) a lower susceptibility to infection by *V. aestuarianus*,
523 and/or (3) a higher resistance to dual infection. Meanwhile, the lower mortality of the 2nR
524 group compared to all other groups indicated that further selection could still be transferred
525 into the diploid and tetraploid broodstocks. For OsHV-1 resistance, mortality was similar
526 between diploids and triploids in *C. gigas* when the same germplasm was used for both ploidy
527 levels, which is in agreement with the fact that OsHV-1 resistance is not substantially altered
528 by triploidization (Dégremont et al., 2016). Nevertheless, triploids had higher mortality when
529 exposed to *V. aestuarianus* even when the same germplasm was used for both ploidies
530 (Azéma et al., 2016; Dégremont et al., 2016). However, further investigations are required to
531 address the importance of selection to improve both OsHV-1 and *V. aestuarianus* resistance
532 for 2n and 4n broodstocks to produce all-triploid stocks of *C. gigas*. Indeed, production of all-
533 triploids usually requires different germplasms that could counteract genetic progress or
534 enhance performances throughout heterosis. Although the progress in the selection for
535 disease-resistant lines is heritable through both the diploid and tetraploid lines and appeared to
536 be additive, as observed in *Crassostrea virginica* resistance to *Perkinsus marinus* and
537 *Haplosporidium nelsoni* (Dégremont et al., 2012), this remains to be demonstrated in *C.*

538 *gigas*, particularly for *V. aestuarianus*.

539

540 During this study, no differences in overall growth were observed among groups, while there
541 were differences in daily growth. These differences were mostly the result of the differences
542 in mortality among groups and are in agreement with previous studies (Dégremont et al.,
543 2005; Evans and Langdon, 2006).

544

545 **5. Conclusion**

546

547 In conclusion, the "plan de sauvegarde" allowed the testing of 104 diploid and triploid batches
548 of *C. gigas* spat in France, mostly produced by commercial hatcheries from 2011 to 2014, in
549 the context of massive mortality related to OsHV-1. The 3nR oysters showed an important
550 decrease in mortality compared to the classically produced 2n and 3n oysters from hatcheries
551 in 2012, and this was related to an increase in resistance to OsHV-1 in both the 2nR and 4nR
552 groups. Unfortunately, this plan was based on a very limited genetic background.
553 Furthermore, the reemergence of *V. aestuarianus* in France since 2012 might have ruined the
554 genetic gain of OsHV-1 resistance in the 3nR oysters in PS3 and PS4 (in 2013 and 2014,
555 respectively).
556 Despite genetic improvement for OsHV-1 resistance, there are a variety of reasons why this
557 translates into variable commercial genetic gain. These include the possible impact of *V.*
558 *aestuarianus*, the difficulty in setting up commercial-scale trials with good genetic
559 representation, the changing levels of genetic improvement in both the 3nR group and the
560 commercial groups, and the lack of broodstock genetic variation where small numbers of
561 males were used. The "plan de sauvegarde" was conducted for four years starting from the
562 few selected lines available in 2009 in order to provide oyster farmers with improved spat.

563 Further experiments are required to unravel the mechanisms involved in the transfer of
564 genetic progress for resistance to OsHV-1 and *V. aestuarianus* from diploids to tetraploids for
565 the production of selected all-triploid oysters.
566
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580
581

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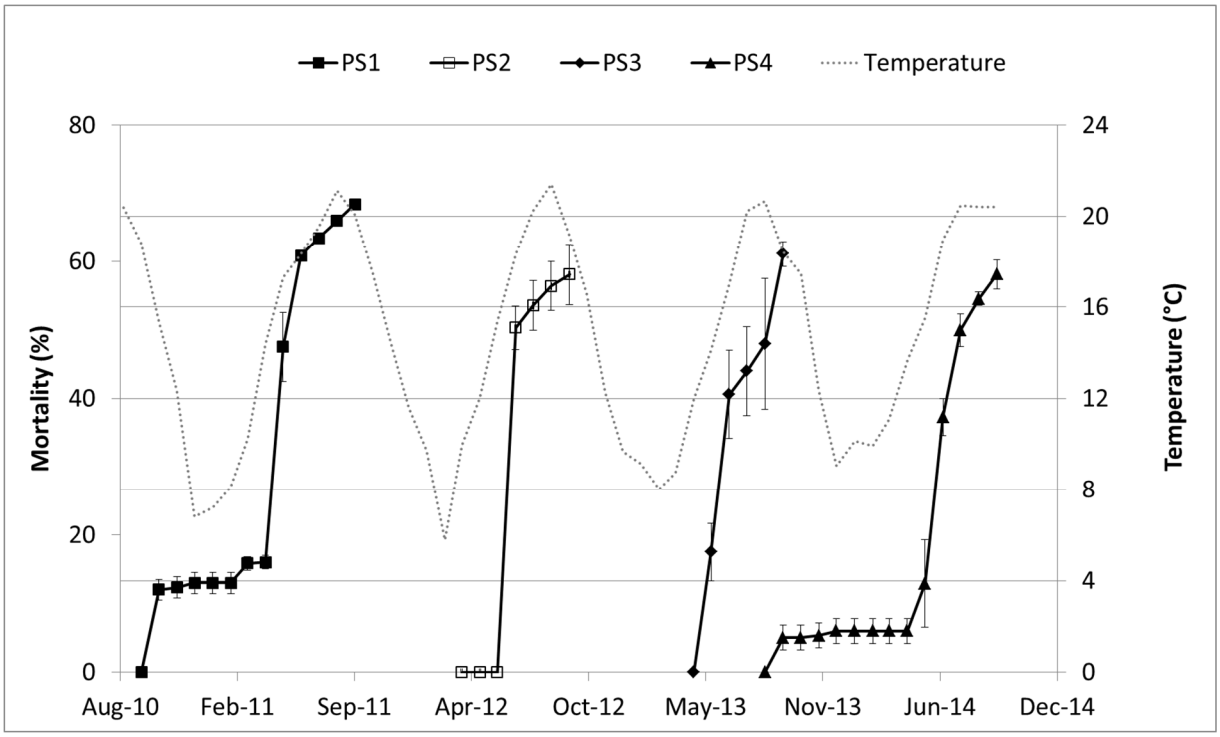

Figure 1 Mean cumulative mortality (±SD among sites) for the four sets (PS1 to PS4) and monthly seawater temperature from August 2010 to September 2014

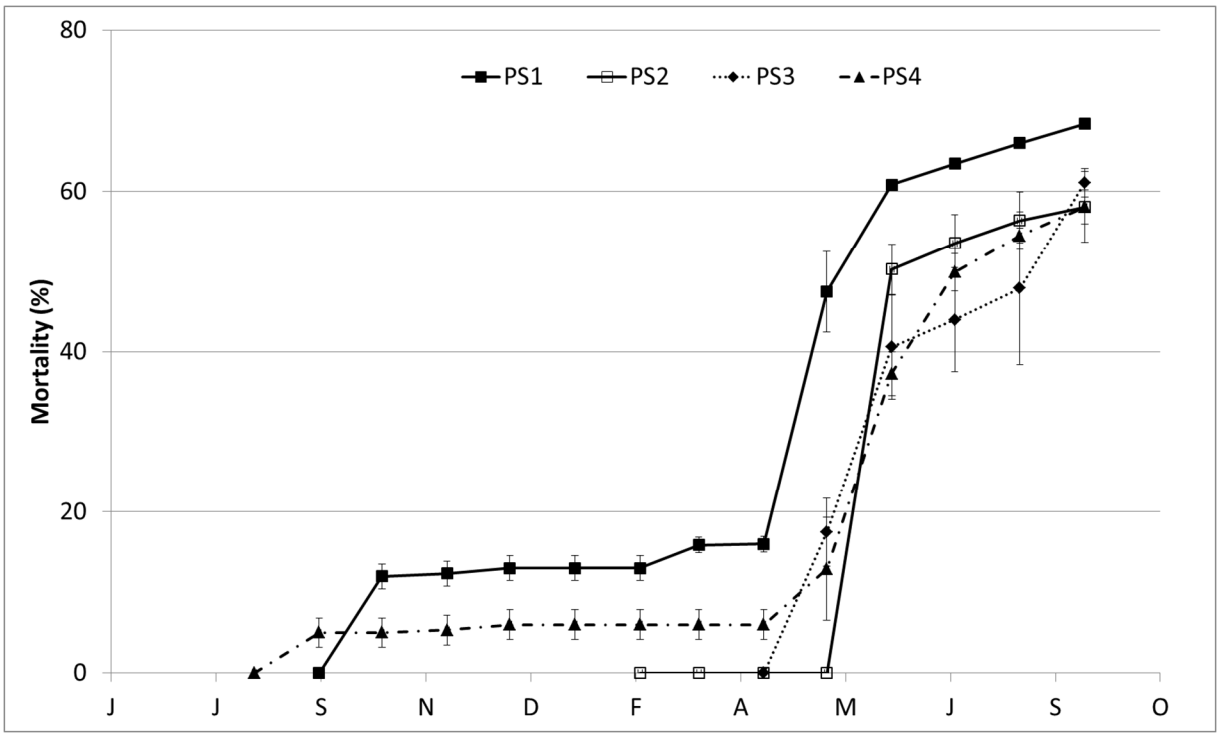

Figure 2 Mean mortality (±SD among sites) for the four sets (PS1 to PS4) from deployment until endpoint in September. Some batches of PS1 and PS4 were deployed into the field the same year of their production while most of the others were deployed the following year.

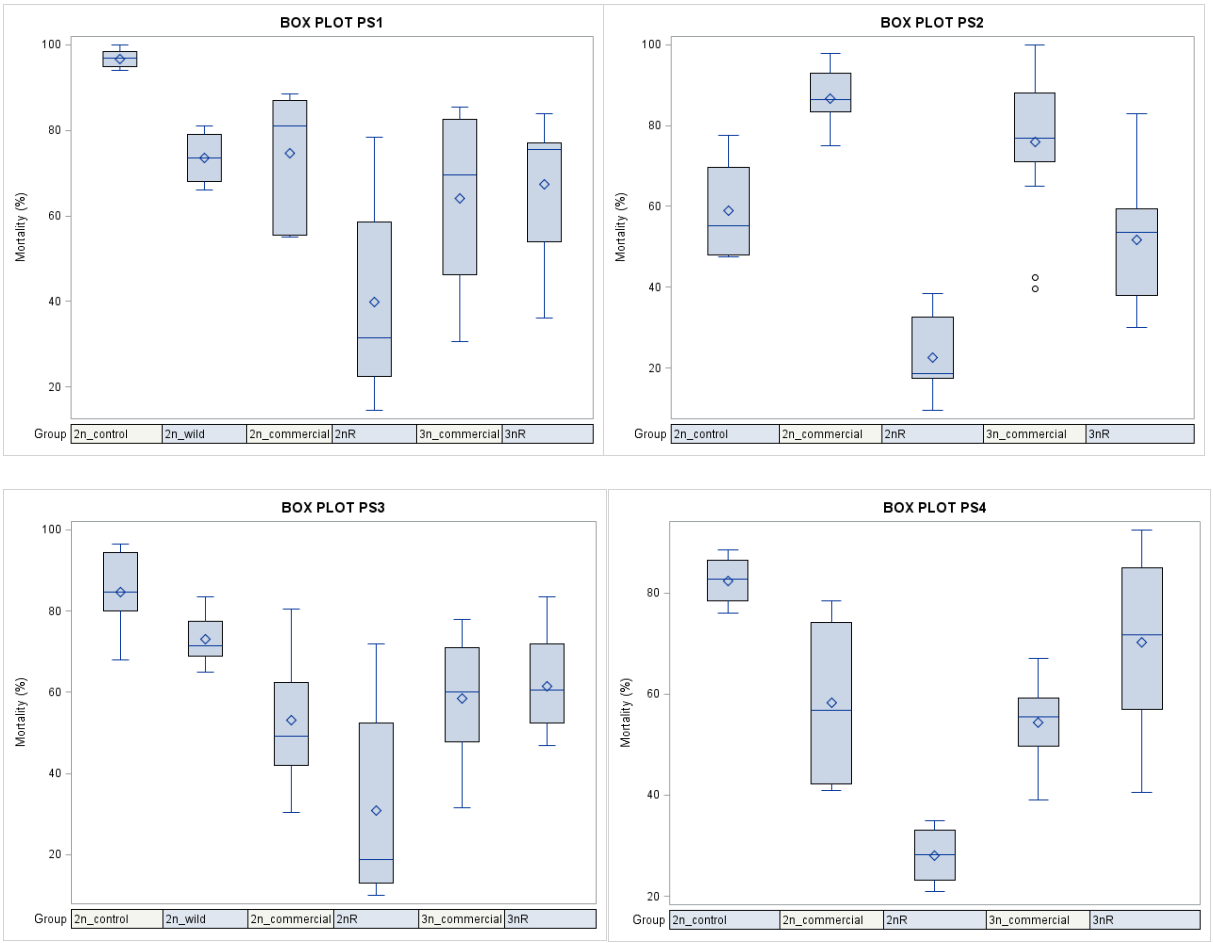

Figure 3 Box plots of the mortality at endpoint of each group for each PS.

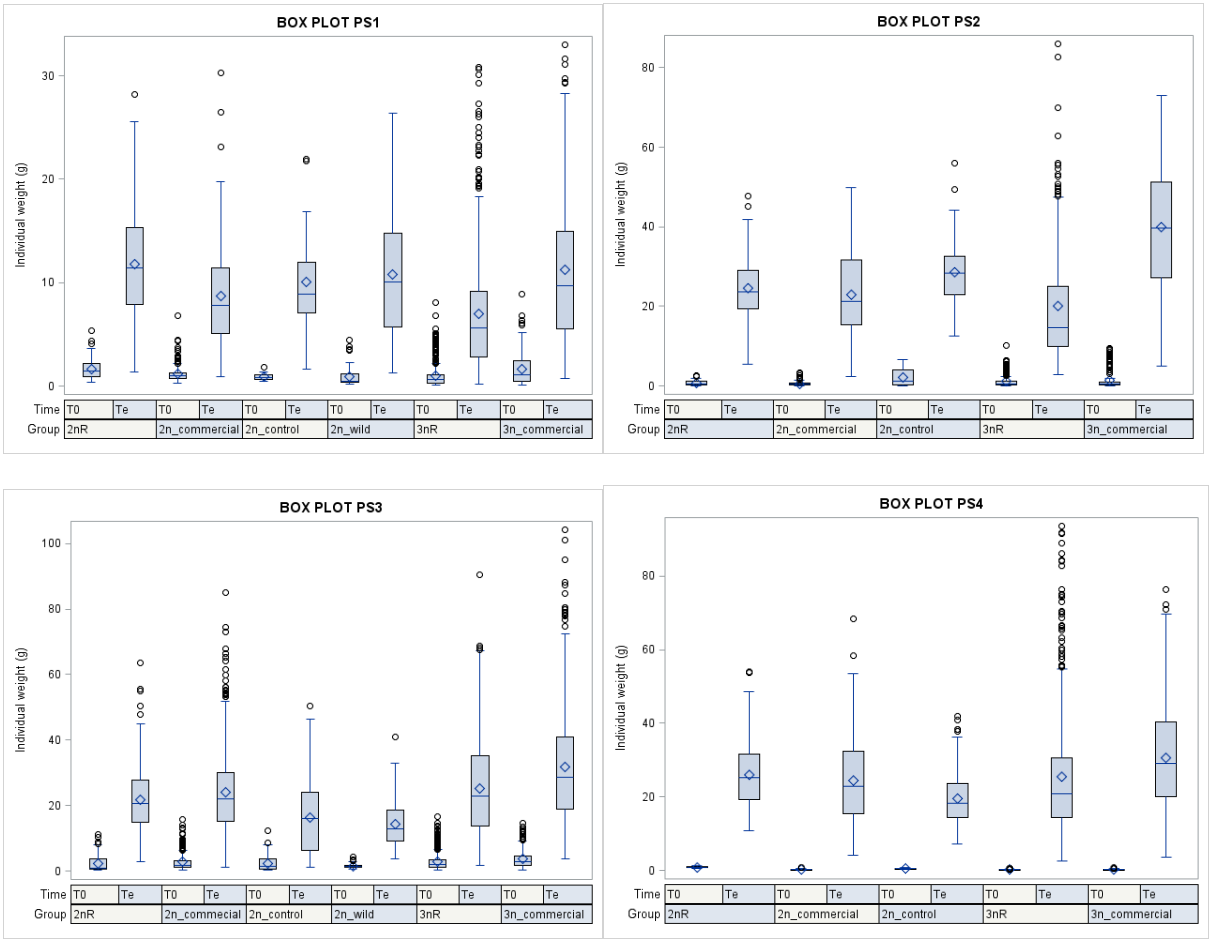

Figure 4 Box plots of the individual weight (g) at deployment (T0) and endpoint (Te)of each group for each PS.

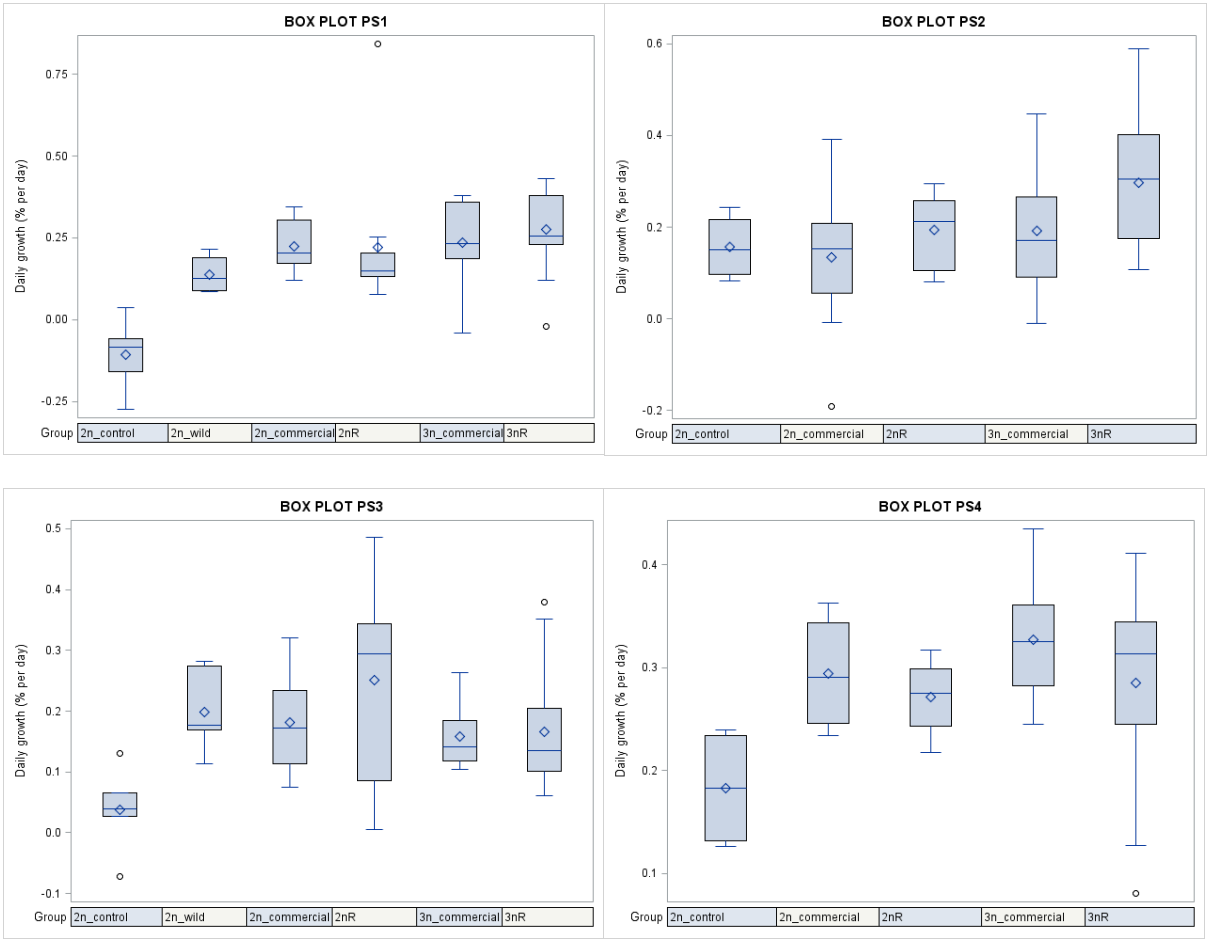

Figure 5 Box plots of the daily growth (% per day) from deployment to endpoint of each group for each PS.

Table 1: Summary of the four Plan de sauvegarde (PS)

PS number	Date of production	Date of deployment	Date of Endpoint	Number of batches per group					
				2n-commercial	3n-commercial	3nR-commercial	2n-control	2nR-control	2n-wild caught
PS1	3/2010 - 9/2010	9/2010 - 3/2011	9/2011	3	4	8	5	5	2
PS2	3/2010 - 9/2011	3/2012	10/2012	5	7	9	2	3	
PS3	5/2012 - 9/2012	4/2013	9/2013	6	6	11	3	3	3
PS4	3/2013 - 7/2013	8/2013 - 2/2014	9/2014	2	6	7	2	2	
Total				16	23	35	12	13	5

Table 2: Quantification of OsHV-1 DNA for live oysters at deployment (T0) and endpoint and for moribund oysters during mortality events for each PS, and quantification of *V. aestuarianus* for moribund oysters sampled for the PS4

			OsHV-1				*V. aestuarianus*		
PS	Date of sampling	Type of oysters	Number of positive	Number sampled	Prevalence (%)	Copies of DNA per mg of fresh oyster tissue[1]	Number of positive	Prevalence (%)	Copies of DNA per mg of fresh oyster tissue[1]
1	T0	live	29	325	9	1E+02			
	May	moribund	44	44	100	9E+05			
	Endpoint	live	98	234	42	1E+03			
2	T0	live	59	260	23	2E+02			
	May	moribund	236	237	100	2E+04			
	Endpoint	live	24	233	10	2E+02			
3	T0	live	3	320	1	4E+02			
	May	moribund	92	103	89	1E+07			
	Endpoint	live	62	314	20	2E+05			
4	T0	live	35	451	8	7E+06			
	May	moribund	112	116	97	8E+07	14	12	1E+07
	July	moribund	33	39	84	5E+08	26	67	3E+06
	Endpoint	live	216	390	55	5E+06			

[1] the value is the mean of the quantities detected only for the positive oysters.

Table 3: Final mean mortality per group for each of the four Plan de sauvegarde (PS)

PS number	2n-control	2n-wild caught	2n-commercial	3n-commercial	3nR-commercial	2nR-control
PS1	96.7 ± 2.1	73.5 ± 6.8	74.6 ± 15.4	64.1 ± 21.9	67.3 ± 15.1	40.0 ± 22.3
PS2	58.9 ± 14.1		86.8 ± 7.2	75.9 ± 17.9	51.6 ± 15.5	22.5 ± 10.8
PS3	84.8 ± 10.4	73.0 ± 6.7	53.0 ± 15.6	58.4 ± 14.9	61.5 ± 10.3	30.8 ± 25.4
PS4	82.5 ± 5.3		58.3 ± 18.8	54.4 ± 7.9	70.3 ± 17.6	28.1 ± 6.1
Total	85.0 ± 15.3	73.2 ± 6.3	68.3 ± 19.9	63.7 ± 17.7	62.0 ± 15.8	32.0 ± 19.6

Table 4: Pairwise difference of mortality between the 3nR group and the other groups and odd ratios of parameter estimates

							95% confidence limit	
PS	Group	Group	Estimate	StdErr	*P*	OddsRatio	Lower	Upper
1	2n-control	3nR-commercial	2.74	0.49	<0.01	15.6	5.6	43.3
1	2n-wild	3nR-commercial	0.27	0.66	0.69	1.3	0.3	5.1
1	2n-commercial	3nR-commercial	0.43	0.56	0.45	1.5	0.5	5.0
1	2nR-control	3nR-commercial	-1.23	0.47	0.02	0.3	0.1	0.8
1	3n-commercial	3nR-commercial	-0.09	0.51	0.87	0.9	0.3	2.6
2	2n-control	3nR-commercial	0.32	0.66	0.63	1.4	0.3	5.4
2	2n-commercial	3nR-commercial	1.92	0.48	<0.01	6.8	2.5	18.5
2	2nR	3nR-commercial	-1.37	0.56	0.02	0.3	0.1	0.8
2	3n-commercial	3nR-commercial	1.35	0.43	0.01	3.9	1.6	9.5
3	2n-control	3nR-commercial	1.31	0.42	<0.01	3.7	1.6	8.7
3	2n-wild	3nR-commercial	0.53	0.41	0.21	1.7	0.7	4.0
3	2n-commercial	3nR-commercial	-0.34	0.32	0.30	0.7	0.4	1.4
3	2nR-control	3nR-commercial	-1.46	0.41	<0.01	0.2	0.1	0.5
3	3n-commercial	3nR-commercial	-0.13	0.32	0.69	0.9	0.5	1.7
4	2n-control	3nR-commercial	0.59	0.54	0.30	1.8	0.6	5.7
4	2n-commercial	3nR-commercial	-0.62	0.54	0.27	0.5	0.2	1.7
4	2nR-control	3nR-commercial	-1.94	0.54	<0.01	0.1	0.0	0.5
4	3n-commercial	3nR-commercial	-0.81	0.37	0.05	0.4	0.2	1.0
All PS	2n-control	3nR-commercial	1.65	0.31	<0.01	5.2	2.8	9.6
All PS	2n-wild	3nR-commercial	0.48	0.44	0.27	1.6	0.7	3.8
All PS	2n-commercial	3nR-commercial	0.39	0.28	0.16	1.5	0.9	2.6
All PS	2nR-control	3nR-commercial	-1.40	0.30	<0.01	0.3	0.1	0.5
All PS	3n-commercial	3nR-commercial	0.14	0.25	0.57	1.2	0.7	1.9