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Interaction between toxic dinoflagellate *Alexandrium catenella* exposure and disease associated with herpesvirus OsHV-1µVar in Pacific oyster spat *Crassostrea gigas*

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Abstract :

Blooms of toxic dinoflagellates can co-occur with mass mortality events associated with herpesvirus OsHV-1 µVar infection that have been decimating Pacific oyster *Crassostrea gigas* spat and juveniles every summer since 2008 in France. This study investigated the possible effect of a harmful dinoflagellate, *Alexandrium catenella*, a producer of Paralytic Shellfish Toxins (PSTs), upon the oyster spat–herpesvirus interaction. Oyster spat from a hatchery were challenged by cohabitation with oysters contaminated in the field with OsHV-1 µVar and possibly other pathogens. Simultaneously, the oysters were exposed to cultured *A. catenella*. Infection with OsHV-1 µVar and PST accumulation were measured after 4 days of experimental exposure.

Exposure to *Alexandrium catenella* modified the host–pathogen interaction by reducing prevalence of OsHV-1 µVar infection. In addition, oysters challenged with OsHV-1 µVar and possibly other pathogens from the environment accumulated smaller amounts of PSTs than unchallenged oysters. Three possible mechanisms are suggested by these results: (i) possible direct interactions between *A. catenella* and herpesvirus (or associated pathogens) could reduce viral transmission and algal availability for oyster consumption; (ii) oyster feeding behavior or digestive functions may have been altered, thus decreasing both uptake of viral particles and consumption or digestion of toxic algae and consequent toxin accumulation; (iii) immuno-activation by *A. catenella* could enhance defense efficiency against OsHV-1 µVar infection. These findings suggest further research on relationships between OsHV-1 µVar and toxic dinoflagellates and their combined effects upon disease transmission and proliferation processes,

as well as on oyster physiological and immunological involvement in this complex, tripartite interaction.

Highlights

► Exposure to *A. catenella* and challenge by cohabitation with OsHV-1-infected oysters. ► *catenella* exposure for 4 days decreased OsHV-1 prevalence. ► Challenge by cohabitation with OsHV-1-infected oysters decreased toxin accumulation.

Keywords : Crassostrea gigas, OsHV-1 µVar, Harmful algal blooms, Alexandrium, Host–pathogen interaction, PST accumulation

1 Introduction

Since 2008, French oyster production has been diminished by massive mortality events that have decimated *Crassostrea gigas* spat (<12 months old) and juveniles (12-18 months old), when seawater temperature exceeds 16°C. These mortality events decimate 40 to 100% of the spat, depending upon location (Cochennec-Laureau et al., 2011; EFSA Panel on Animal Health and Welfare, 2010; Fleury and Bédier, 2013; Fleury, 2014). Other countries in Europe, as well as New Zealand, Australia and Korea also recently experienced massive spat and juvenile oyster mortality events, which were associated with microvariants of ostreid herpesvirus 1 (OsHV-1) (EFSA Panel on Animal Health and Welfare, 2010; Hwang et al., 2013; Jenkins et al., 2013; Martenot et al., 2011; Paul-Pont et al., 2014; Renault et al., 2012). The microvariant genotype detected in France, designated as OsHV-1µVar, was found also in Ireland, the UK, Spain, the Netherlands, New Zealand and Australia (EFSA Panel on Animal Health and Welfare, 2010; Gittenberger et al., 2015; Jenkins et al., 2013; Renault et al., 2012; Roque et al., 2012; Segarra et al., 2010). A causal relationship between OsHV-1µVar and mortality events has been postulated (Schikorski et al., 2011a, 2011b), although bacterial strains from the *Vibrio splendidus* clade and *Vibrio aesturianus* also could be involved (EFSA Panel on Animal Health and Welfare, 2010; Garnier et al., 2007; Gay et al., 2004), and environmental factors and oyster physiological condition may play roles in disease severity and oyster susceptibility to the disease (Pernet et al., 2014, 2012; Petton et al., 2013).

Concurrent with these mortality events, during spring and summer, toxic dinoflagellates *Alexandrium minutum* and *Alexandrium catenella* recurrently bloom along the French coasts (Amouroux et al., 2013; Belin et al., 2015; Chapelle et al., 2013; Fleury and Bédier, 2013; Fleury, 2014; Lilly et al., 2002; REPHY; RESCO). Temperature thresholds above which blooms usually are reported are in the range 15-18°C (Chapelle et al., 2013, 2007; Collos et

al., 2009; Laabir et al., 2011), corresponding to temperature at which mortality events are observed (>16°C). For example, *A. minutum* blooms have occurred in the Bay of Brest (N.W. of France) every summer since 2012, with a maximum concentration of 42×10^6 cells L^{-1} detected in 2012 (Chapelle et al., 2013; http://envlit.ifremer.fr/var/envlit/storage/documents/parammaps/phytoplancton/index.html); and 69% cumulative oyster spat mortality was recorded there at the end of the summer this year. In the Mediterranean sea, *A. catenella* blooms occur recurrently, including in the Thau Lagoon (S. of France) where, for example, 2.5×10^5 cells L^{-1} were detected in June 2011 (Belin et al., 2012), concurrent with oyster spat mortality events mainly in May and June 2011 (for mortality reports in France by year and site, see RESCO website, http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux). Both these *Alexandrium* species can produce Paralytic Shellfish Toxins (PSTs). Bivalves, as filter-feeders, can accumulate PSTs, leading to toxicity for human shellfish consumers and thus resulting in temporary shellfish harvest prohibitions. Species of the genus *Alexandrium* can also produce extracellular compounds with allelopathic, hemolytic, ichtyotoxic, and oxidative properties (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). Interactions between bivalves and *Alexandrium* sp. – and thus PSTs and extracellular compounds – have been examined in many studies. Alterations of physiological processes and tissue integrity have been reported in bivalves after a few hours to a few (4-9) days of exposure to *Alexandrium* sp. cells (10^2-10^3 cell mL^{-1}) (Cucci et al., 1985; Gainey and Shumway, 1988a, 1988b; Galimany et al., 2008a; Haberkorn et al., 2010b; Landsberg, 2002; Lassudrie et al., 2014; Medhioub et al., 2012; Shumway, 1990). Hemocytes, which are involved in many functions including immunity, also are affected by *Alexandrium* sp. within a few days of exposure (Galimany et al., 2008a; Haberkorn et al., 2010a; Hégaret et al., 2007). By altering cell functions involved in immune response, physiological processes and tissue integrity in bivalves, *Alexandrium* sp.

93 may modify shellfish susceptibility to pathogens. Indeed, Lassudrie et al. (2015) reported that
94 a three-week exposure to *Alexandrium fundyense* (a PST producer) resulted in modified
95 immune functions associated with higher susceptibility to infection with the protozoan
96 parasite *Perkinsus marinus* in trematode-infested Eastern oysters. On the contrary, da Silva et
97 al. (2008) reported a decrease in intensity of *Perkinsus olseni* infection in Manila clams
98 exposed to the toxic dinoflagellate *Karenia selliformis* for 2 weeks, which was shown to result
99 from direct toxicity of algae to *P. olseni*. Similarly, Hégaret et al. (2010) suggested a toxic
100 effect of the dinoflagellate *Prorocentrum minimum* upon the protistan Quahog Parasite
101 Unknown (QPX) in northern quahog clams. Thus, toxic algal exposure may modify the host-
102 pathogen interaction, either by affecting host physiological or immune status, or through
103 direct toxicity to the pathogen.

104 Despite the importance of OsHV-1µVar in oyster mortality events in France and the repeated
105 co-occurrence with harmful algal blooms, to the best of our knowledge, the effect of toxic
106 algal exposure upon the oyster spat – herpesvirus interaction has not been described.

107 The aim of this study was, thus, to assess if and how exposure to the toxic dinoflagellate, *A.*
108 *catenella* could modulate the host-pathogen interaction, i.e. oyster spat – herpesvirus
109 interaction. For this purpose, oyster spat naïve from herpesvirus (Specific Pathogen-Free,
110 SPF) were exposed simultaneously to cultured *A. catenella* (at realistic concentrations
111 compared to the field) and challenged with herpesvirus and possible other pathogenic agents
112 associated with mortality events.

2 Material and methods

2.1 Algal cultures

Tisochrysis lutea (Bendif & Probert) (T-*Iso*) was used as the diet during acclimation and maintenance stages at 5×10^5 cells mL^{-1}. T-*Iso* was cultured in 300-L cylinders containing seawater enriched with Conway medium (Walne, 1966) at 20°C with continuous light (200 µmol photons m^{-2} s^{-1}). T-*Iso* was harvested after 3 to 5 days of growth, at a cell density approaching 1×10^7 cells mL^{-1}.

The dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) strain VGO676, a PST producer (Lassus et al., 2007) isolated in 2003 from Thau lagoon (France), was used for toxic algal exposure, and *Heterocapsa triquetra* (Ehrenberg) Stein, strain HT99PZ (isolated from Penzé Bay, France in 1999) was used as a control, non-toxic dinoflagellate. Both strains were provided by the Phycotoxin laboratory, Ifremer, Nantes (France). Both dinoflagellate cultures were grown in L1 medium (Guillard and Hargraves, 1993) at 17°C with a light:dark cycle of 12:12h and were harvested during exponential growth phase at a cell density approaching 5×10^4 cells mL^{-1}.

Algal cell densities were determined by counts using Malassez and Nageotte cells under a light microscope.

2.2 Specific Pathogen-Free (SPF) oysters

The Pacific oysters, *Crassostrea gigas* (Thunberg), used in this study came from a single cohort produced in March 2012 in the Argenton Ifremer facilities (France) following a

134 standardized process to obtain OsHV-1-free diploid-oysters described by Petton et al. (2013).
135 Screening for OsHV-1 DNA (i.e. OsHV-1 reference as well as OsHV-1µVar) was conducted
136 by qPCR (following the standard procedure described in Pépin et al., 2008) once during D-
137 larval stage and again at 3 months of age following thermal challenge; all results were
138 negative for this cohort (analyses by IDHESA, Quimper, France). At the time the experiment
139 began (July 2012), oysters were 4 months old, measured 23.1 ± 0.2 mm length (mean ± SE),
140 and weighed 1.45 ± 0.3 g (total wet weight; mean ± SE).

141

142 *2.3 OsHV-1 contamination by field-exposure*

143 On June 18th, 2012, a subsample of SPF oyster spat (n=1000) was transferred into the Bay of
144 Brest, at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W), in a farming area with
145 recurrent mass mortality of oysters (Fleury and Bédier, 2013; Fleury, 2014; François et al.,
146 2010). This time corresponded with the highest peak of spat mortality in this location in 2012
147 (30-34% instantaneous mortality) (RESCO,
148 http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-
149 2012/Mortalite-par-site-et-par-classe-d-age). After 3 weeks in the field (i.e. July 2nd, 2012),
150 detection of OsHV-1 DNA in experimental oysters was confirmed by qPCR (following the
151 procedure described below), and oysters were transferred to the experimental facilities.
152 Oysters were held for three days before the beginning of the experiment in two tanks supplied
153 with an open flow of 1-µm-filtered seawater treated with UV, at 20°C, and fed continuously
154 with T-*Iso* at 3-5 × 10^5 cell mL^{-1}. One hundred and eighty field-exposed oysters then were
155 used to challenge SPF oysters by cohabitation, as described below, and were also analyzed
156 (designated as "field-exposed oysters"). The same number of SPF oysters was maintained in

two other tanks in the same conditions, and these oysters were used for cohabitation with unchallenged oysters.

2.4 Experimental design

On June 25th, 2012, SPF oysters were distributed in 15-L tanks (30 oysters per tank, 12 tanks) and acclimated for 10 days being fed T-*Iso*. At the end of this acclimation period, on July 5th (T_0), 30 field-exposed oysters per tank, held in a net, were added to six of these tanks. The SPF oysters that were thus maintained in cohabitation with the field-exposed oysters were designated as “challenged”. In the six other tanks, 30 other SPF oysters per tank, held in a net, were added to establish the same number of oysters in each tank. The oysters in these tanks were designated as “unchallenged”. In addition, 3 “challenged” tanks and 3 “unchallenged” tanks were exposed continuously to 3.5×10^2 cells mL^{-1} of the toxic dinoflagellate *A. catenella*; whereas, the other tanks were exposed to the same concentration of the control, non-toxic dinoflagellate, *H. triquetra.*

Experimental design is summarized in Figure 1. Four experimental conditions were used: *A. catenella* exposure and challenged oysters (condition 1); *A. catenella* exposure and unchallenged oysters (condition 2); non-toxic algal exposure and challenged oysters (condition 3); non-toxic algal exposure and unchallenged oysters (condition 4).

A similar cohabitation design previously has been demonstrated to successfully infect SPF oysters with the pathogenic agents responsible for mass mortality, including OsHV-1µVar (Petton et al., 2013).

During the entire experiment, 1-µm-filtered and UV-treated seawater was supplied to the tanks (10-15 mL min^{-1}, i.e. one tank renewal every 24h) with aeration at 20°C, a temperature favorable for OsHV-1µVar transmission (Petton et al., 2013).

2.5 Sampling

Oysters were sampled at the end of the acclimation period, i.e. before the beginning of the experiment (T_0), and after four days (T_4). At T_0, 12 field exposed-oysters, 12 SPF oysters from "unchallenged" tanks (2 per tank), and 12 SPF oysters from "challenged" tanks (2 per tank) were sampled. At T_4, 72 unchallenged oysters (12 per tank), 72 challenged oysters (12 per tank), and 72 field-exposed oysters from "challenged" tanks (12 per tank) were sampled.

Mantle and digestive gland were sampled for herpesvirus and toxin quantification, respectively, and tissue samples were frozen immediately in liquid nitrogen before being stored at -80°C prior to quantification.

Oyster mortality was checked daily. Dead oysters were removed from the tanks, and mantle and digestive gland were dissected, when the degradation stage of the tissue allowed it, and stored at -80°C for further analyses.

Not all oysters sampled were used for each analysis (see sections below).

2.6 OsHV-1 DNA quantification

OsHV-1 DNA was quantified in the mantle at the end of the acclimation period (T_0) (before algal exposure and cohabitation challenge) in 2 SPF oysters per tank, which were designated to be challenged (i.e. 12 oysters) and 1 oyster per tank designated to remain unchallenged (i.e. 6 oysters) to confirm oysters were not contaminated at the beginning of the experiment. Additionally, 11 field-exposed oysters were also analyzed at T_0 to confirm that contamination occurred in the field. After four days of the experiment, OsHV-1 quantification was performed in 36 and 35 challenged oysters exposed to *H. triqu*etra and *A. catenella*, respectively (11-12 oysters per tank), in 23 and 24 field-exposed oysters exposed to *H. triqu*etra and *A. catenella*, respectively (7-8 oysters per tank); and in 3 unchallenged oysters per algal condition (one per tank) as these oysters were not expected to be contaminated. Analysis also was performed for 25 of the 27 field-exposed oysters and for all the 5 challenged oysters that died over the course of the experiment.

DNA extraction was performed with the QIAamp DNA Mini Kit (QIAgen), analyzing 20 mg of wet mantle, following the manufacturer instructions. 100 µL of molecular biology grade water (DNase-free) were used for elution. Nucleic acid concentration was measured immediately with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (conversion factor: 1 OD = 50 µg mL^{-1} DNA) at 260 nm and adjusted at 3 ng μL^{-1} after purity was checked using the 260 / 230 nm and 260 / 280 nm ratios.

A standard protocol was followed to quantify OsHV-1 DNA (OsHV-1 reference as well as OsHV-1µVar) using qPCR (Pépin, 2013; Pépin et al., 2008) with the HVDP-F – HVDP-R primer pair (forward, HVDP-F 5'-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3', reverse, HVDP-R 5'-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3'), targeting the OsHV-1 polymerase sequence. Each reaction was performed in triplicate and carried out in a final volume of 15 µL containing HVDP-F and HVDP-R primers at final concentrations of 5 µM,

7.5 µL of IQ SYBR Green Supermix and 5µL of DNA samples diluted at 3 ng μL^{-1}. Each run included a no-template control (water), a positive control (DNA from OsHV-1-infected oyster), and six standards (from 10^5 to 10^0 OsHV-1 copies μL^{-1}), prepared by successive, ten-fold dilutions of a stock solution of OsHV-1 genomic DNA at 5×10^6 copies μL^{-1} extracted from purified virus particles (Le Deuff and Renault, 1999). The standard curve obtained was used to calculate the percentage of amplification efficiency (% E) described in (1), which was contained between 90% and 110%, and quantification of the samples was determined by comparing Ct values.

(1) $\% E = (10^{-1/a} - 1) \times 100$, with a = slope of the linear regression line calculated from Ct = f($\log_{10}$ of dilution).

An MyIQ2 Thermocycler (Biorad) was used with the following thermal profile: 1 cycle of enzyme activation (95°C, 3min.), 40 cycles of amplification/detection (95°C, 30s; 60°C, 1min.; 72°C, 1min), and a final step for melting temperature curve analysis (80 cycles, 95°C to 55°C, decreasing the temperature by 0.5°C after each cycle, 10s). The specificity of the PCR products was checked systematically with the melting temperature (Tm) value calculated from the dissociation curve.

Results were expressed as number of OsHV-1 DNA copies ng^{-1} total DNA. In addition, quantification expressed as OsHV-1 DNA copies mg^{-1} of wet mantle was determined. Correlation between both ways of expressing OsHV1 DNA quantification was verified (Pearson product moment correlation, $p<0.001$, correlation coefficient = 0.98, n=269).

The following criteria were used to consider OsHV-1 detection as positive: Ct<38 and a Tm corresponding to OsHV-1 DNA amplicon (77.5°C). When OsHV-1 DNA concentration in

243 samples considered as positive were below the detection limit (i.e. $Ct_{sample}>Ct_{standards}$), a
244 numerical value of 0 was given for quantification.

245

246 *2.7 Identification of OsHV-1µVar*

247 The standard qPCR method used to quantify OsHV-1 DNA cannot differentiate the OsHV-1
248 reference from OsHV-1 µVar genotype. Therefore, sequencing of 2 regions of OsHV-1 DNA
249 amplified with C2/C6 and IA1/IA2 primer pairs was performed following the procedure
250 described by Segarra et al. (2010). This analysis was performed on DNA extracted from the
251 mantle of 10 individuals, including both field-exposed and challenged oysters, exposed to
252 both algal treatments, alive and dead. Comparison of the nucleotide sequences obtained with
253 OsHV1 reference and OsHV1µVar sequences confirmed the sole presence of the OsHV1µVar
254 genotype in the samples. Considering both this analysis and the report of OsHV-1µVar having
255 replaced OsHV-1 reference genotype since 2009 in spat at French rearing sites suffering mass
256 mortality (including Bay of Brest), (François et al., 2010; Petton et al., 2013; Renault et al.,
257 2012), the OsHV-1 genotype in the present study was considered to be OsHV-1µVar.

258

259 *2.8 Toxin accumulation*

260 Three SPF oysters at T_0 and 3 oysters exposed to the non toxic *H. triquetra* and not previously
261 held in the field were tested to ensure that no PST was detected. 23 challenged and 23
262 unchallenged-oysters (7-8 oysters per tank) were analyzed after 4 days of *A. catenella*-
263 exposure. Twelve field-exposed oysters also were analyzed for toxin accumulation at T_0, as

264 well as 12 field-exposed oysters from each algal treatment at T_4 (4 oysters per tank), PST
265 analysis also was performed after *A. catenella* exposure on 8 of the 12 dead field-exposed
266 oysters, and in the 3 dead oysters unexposed to the field.

267 PST accumulation was assessed using the Saxitoxin (PSP) ELISA kit (Abraxis), as in
268 Lassudrie et al. (2015). PST extraction was performed following manufacturer instructions:
269 digestive gland tissue was homogenized in HCl 0.1 M (1:1, w:v) using a Precellys®24 bead-
270 grinder and then boiled for 5 min, leading to acid hydrolysis that can induce chemical
271 conversion of some PST analogues to STX (Vale et al., 2008). Indeed, this ELISA assay
272 recognizes mostly STX, and other PSTs only to varying degrees (cross-reactivities of 100%
273 for STX and <30% for other PSTs). Therefore, PST content was expressed as µg STX kg^{-1} of
274 wet digestive gland weight.

275

276 *2.9 Statistical analyses*

277 For all statistical tests, absence of difference between replicate tanks was first verified before
278 comparing different conditions.

279 To compare cumulative mortality curves (Kaplan Meier method) between conditions, a Log-
280 rank (Mantel-Cox) test followed by multiple comparison tests with Holm-Bonferroni
281 correction was used.

282 Difference between OsHV-1 prevalence in live oysters according to algal exposure was tested
283 with a Chi-square test.

In live oysters, effect of algal treatment (*A. catenella* or *H. triquetra*), and effect of time of sampling in field-exposed oysters were tested upon OsHV-1 DNA quantification with a *t*-test. OsHV-1 DNA data were $\log_{10}$ (X + 1) transformed to obtain normality of residuals and homogeneity of variances. A $\log_{10}$ scale was used in graphical representation of OsHV-1 DNA data.

Differences in PST accumulation in digestive glands were tested with a Mann-Whitney test to compare challenged and unchallenged oysters, and with a Kruskal-Wallis test followed by a Nemenyi-Damico-Wolfe-Dunn (NDWD) post-hoc test to compare field-exposed oysters at T_0, after 4 days of exposure to *A. catenella* and after 4 days of exposure to *H. triquetra*.

Differences were considered significant when $p<0.05$. Statistical analyses were performed using Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA), R version 2.15.1 (R Core Team, 2012) and GraphPad Prism. All values were expressed as mean ± standard error.

297 **3 Results**

298

299 *3.1 Mortality*

300 Cumulative mortality (Figure 2) accounted for 16.7% and 13.3% of field-exposed oysters
301 after 4 days of exposure to *H. triquetra* and *A. catenella*, respectively. After 4 days of the
302 experiment, 3.3% and 2.2% mortality was found in challenged oysters exposed to *H. triquetra*
303 and *A. catenella*, respectively; whereas 2.8% and 0.6% mortality were found in unchallenged
304 oysters exposed to *H. triquetra* and *A. catenella*, respectively. Cumulative mortality was
305 significantly higher ($p<0.05$) in field-exposed oysters compared to unchallenged oysters fed
306 both *A. catenella* and *H. triquetra*, and challenged-oysters fed *A. catenella*. Cumulative
307 mortality in field-exposed oysters, however, did not differ significantly from cumulative
308 mortality in challenged oysters fed *H. triquetra*.

309

310 *3.2 OsHV-1 DNA detection and quantification*

311 Within the dead oysters:

312 OsHV-1 DNA was detected in 100% of the dead oysters, either previously exposed to the field
313 ($1.1 \pm 0.2 \times 10^6$ copies ng^{-1} total DNA, equivalent to $2.5 \pm 0.5 \times 10^9$ copies mg^{-1} of wet
314 mantle, n=25) or challenged ($1.0 \pm 1.0 \times 10^5$ copies ng^{-1} total DNA, equivalent to $2.0 \pm 2.0 \times$
315 10^8 copies mg^{-1} of wet mantle, n=5). In dead, unchallenged oysters, OsHV-1 was detected in 3
316 of 4 oysters, but only at low intensity levels (between 6.6×10^0 and 2.9×10^2 copies ng^{-1} total
317 DNA).

318 Within live oysters:

319 OsHV-1 DNA was not detected in SPF oysters sampled at the end of the acclimation period
320 (T_0; n=12 oysters from tanks designated to be challenged and n=6 oysters from tanks
321 designated to remain unchallenged) or in unchallenged oysters sampled after 4 days of
322 experimental treatment (n=6).

323 OsHV-1 DNA was detected in all field-exposed oysters sampled at T_0 (n=11), with a mean of
324 $4.9 \pm 3.0\ 10^5$ copies ng^{-1} total DNA in mantle ($1.2 \pm 0.8\ 10^9$ copies mg^{-1} of wet mantle) (N.B:
325 field-exposed oysters were unintentionally exposed to a natural, unexpected *A. minutum*
326 bloom). In field-exposed oysters, prevalence did not decrease significantly between T_0 and
327 after 4 days of experiment (98%, n=47) and did not vary significantly with experimental algal
328 exposure. OsHV-1 infection intensity in field-exposed oysters, however, decreased
329 significantly between T_0 and after 4 days of *A. catenella* exposure ($p<0.05$) ($9.6 \pm 9.2 \times 10^2$
330 copies ng^{-1} total DNA, or $2.7 \pm 2.5 \times 10^6$ copies mg^{-1} of wet mantle, n=23), but not after *H.*
331 *triquetra* exposure ($1.4 \pm 0.8 \times 10^5$ copies ng^{-1} total DNA or $4.4 \pm 2.5 \times 10^8$ copies mg^{-1} of
332 wet mantle, n=23). Therefore, OsHV-1 intensity level was significantly lower in field-exposed
333 oysters exposed to *A. catenella* compared to field-exposed oysters exposed to *H. triquetra*
334 ($p<0.05$).

335 In challenged oysters, at T_4, OsHV-1 prevalence was significantly lower ($p<0.05$) after
336 exposure to *A. catenella* (29%) compared to *H. triquetra* (58%, Figure 3A). OsHV-1 infection
337 intensity (i.e. OsHV-1 DNA quantification considering only oysters detected positive to
338 OsHV-1) tended to be lower in *A. catenella*-exposed oysters with $7.0 \pm 5.6 \times 10^2$ copies ng^{-1}
339 total DNA (or $1.9 \pm 1.5 \times 10^6$ copies mg^{-1} of wet mantle, n=10), compared to $2.8 \pm 2.5 \times 10^4$
340 copies ng^{-1} total DNA (or $6.4 \pm 5.6 \times 10^7$ copies mg^{-1} of wet mantle, n=21) in oysters exposed

to *H. triquetra*, although no significant difference was detected (Figure 3B). Finally, a significantly lower OsHV-1 weighted prevalence (i.e. mean of OsHV-1 DNA quantification in oysters, considering OsHV-1–positive and OsHV-1–negative oysters) was detected in *A. catenella*-exposed oysters ($p<0.05$) ($2.0 \pm 1.6 \times 10^{2}$ copies ng^{-1} total DNA or $5.4 \pm 4.5 \times 10^{5}$ copies mg^{-1} of wet mantle, n=35) compared to oysters exposed to the non-toxic *H. triquetra* ($1.6 \pm 1.5 \times 10^{4}$ copies ng^{-1} total DNA or $3.7 \pm 3.3 \times 10^{7}$ copies mg^{-1} of wet mantle, n=36) (Figure 3B).

3.3 Toxin accumulation

No PST was detected in digestive glands of SPF oysters sampled at T_0 (n=3) and after 4 days exposure to the non toxic dinoflagellate *H. triquetra* (n=3).

In field-exposed oysters, PST level at T_0 was $8.8 \pm 1.3 \times 10^{3}$ µg STX kg^{-1} (n=12) due to a natural, unexpected *A. minutum* bloom. PST level decreased significantly more after 4 days of *H. triquetra* exposure ($2.9 \pm 0.6 \times 10^{3}$ µg STX kg^{-1}; n=12) than after *A. catenella* exposure ($5.3 \pm 0.4 \times 10^{3}$ µg STX kg^{-1}; n=12) ($p<0.01$).

After 4 days of *A. catenella* exposure, significantly lower PST accumulation ($p<0.05$) was detected in digestive gland of challenged oysters ($3.6 \pm 0.6 \times 10^{2}$ µg STX kg^{-1}, n=23) compared to unchallenged oysters ($6.9 \pm 1.1 \times 10^{2}$ µg STX kg^{-1}, n=23) (Figure 4).

PSTs were not detected in the 3 dead oysters exposed to *A. catenella* (2 challenged and 1 unchallenged, dead after 3 days of experiment) that were analyzed, whereas $9.7 \pm 2.1 \times 10^{3}$ µg STX kg^{-1} were detected after *A. catenella* exposure in field-exposed oysters that died

during the experiment (previously exposed to a natural, unexpected *A. minutum* bloom in the field) (n=8).

4 Discussion

Exposure to *A. catenella* was shown for the first time to reduce herpesvirus infection; furthermore, PST accumulation decreased in oysters challenged with the herpesvirus and possibly also other pathogenic agents associated with mortality. These interactions constitute an antagonistic relationship between the virus and the toxic alga when they co-occur. Responses of oysters may result from direct interactions between OsHV-1µVar and *A. catenella* and their toxic compounds, or may be the consequence of immune and physiological responses to the virus and / or the microalga.

Low cumulative mortality (from 2.2% to 16.7% depending upon experimental treatment) was observed in the present study after 4 days of experimental treatment at 20°C. Using a similar cohabitation design, Petton et al. (2013) tested the effect of temperature upon mortality kinetics. Cumulative mortality observed in the present study was in a similar range to that reported by Petton et al. (2013), who observed no mortality in challenged oysters and ~8% in field-exposed oysters after 4 days at 17.5°C. Cumulative mortality observed in our study, however, was lower than that observed after 4 days at 21.9°C by Petton et al. (2013), who reported ~20% mortality in challenged oysters and 40% in field-exposed oysters. These authors also observed higher mortality in oyster spat previously held in the field during mass mortality events than in challenged oysters, which is consistent with our study. All oysters exposed to the field or challenged by cohabitation with field-exposed oysters that died during the experiment were infected with OsHV-1. Some dead, unchallenged oysters contained very low levels of OsHV-1 DNA. Seawater supplied, however, was sterilized by UV treatment,

suggesting that a low cross-contamination between tanks may have occurred through aerosols. The very low intensity detected in dead, unchallenged oysters ($\leq 2.9 \times 10^2$ copies ng^{-1} total DNA), compared with dead, field-exposed or challenged oysters (10^5 to 10^6 copies ng^{-1} total DNA) indicated that death was not related to OsHV-1 infection. Additionally, contamination was not detected in any of the live, unchallenged oysters analyzed throughout the experiment, suggesting a minor impact of putative cross-contamination.

The relatively low infective biomass (i.e. field-exposed oysters) used, and the short cohabitation time limited mortality in challenged oysters (for more information about influence of infective biomass and duration of cohabitation see Petton et al., 2015). These conditions were implemented to allow the study of live oysters containing sub-lethal levels of OsHV-1µVar, based upon results reported by Petton et al. (2013). These authors detected OsHV-1 DNA in 50% of oysters challenged for 3 days at 21.9°C, a result consistent with the 58% detection found in the present study in challenged oysters exposed to the non-toxic dinoflagellate for 4 days, at 20°C.

Exposure to the toxic dinoflagellate *A. catenella*, however, significantly reduced the prevalence of OsHV-1µVar from 58% to 29%. Exposure to *A. catenella* also induced a significant decrease of OsHV-1µVar weighted prevalence and a non significant decrease of the infection intensity in challenged oysters. In field-oysters unintentionally exposed to a natural *A. minutum* bloom prior to the experiment, an experimental *A. catenella* exposure decreased OsHV-1µVar intensity compared to non-toxic algal conditions. These results suggest an effect of *A. catenella:* (i) upon proliferation and virulence mechanisms of OsHV-1µVar, through modulation of oyster immune responses, (ii) upon horizontal transmission of OsHV-1µVar, either through direct interaction between the virus and the algal cells, or by modulating the oyster feeding processes.

411 Proliferation of OsHV-1µVar may have been inhibited by activation of the host immune
412 responses induced by *A. catenella* exposure. Although immune defense against herpesvirus is
413 not well documented, hemocyte-mediated responses appear to be involved, as shown by
414 hemocyte infiltrations (Jenkins et al., 2013) and differentially expressed genes related to
415 hemocyte functions in virus-infected oysters (Green et al., 2014; Jouaux et al., 2013; Renault
416 et al., 2011). Previous studies showed an inflammatory response in bivalves exposed to
417 *Alexandrium* sp., which increased circulating hemocyte concentration, as well as hemocyte
418 infiltration and diapedesis, particularly in the digestive gland but also in the gills and the
419 mantle (Galimany et al., 2008a; Haberkorn et al., 2010a, 2010b; Lassudrie et al., 2014). More
420 numerous hemocytes in these organs that are also targeted by OsHV-1 (Corbeil et al., 2015;
421 Schikorski et al., 2011a) could result in a faster, more efficient defense and accelerate
422 elimination of herpesvirus, possibly through release of humoral factors. For example, α2-
423 macroglobulin molecules are protease inhibitors expressed in hemocytes that play a role in the
424 immune defense (Gueguen et al., 2003; Vaseeharan et al., 2007), and are upregulated with
425 herpesvirus infection in *C. gigas* (Jouaux et al., 2013). Increase in hemocyte apoptosis
426 induced by *A. catenella* (Medhioub et al., 2013) also may play a role, as apoptosis is an
427 important component of immune defense against intra-cellular pathogens by limiting their
428 proliferation (Everett and McFadden, 1999; Sokolova, 2009), and appears to be involved in
429 response to OsHV-1 (Jouaux et al., 2013). Exposure to *Alexandrium* sp. also can increase
430 oxidative stress in bivalves, which could alter the integrity of the lipid-containing envelope of
431 herpesvirus particles. In fact, exposure to *Alexandrium* spp. modulated antioxidant
432 mechanisms (enzyme activity or gene expression) in clams and oysters (Estrada et al., 2007;
433 Lassudrie et al., 2014; Romero-Geraldo and Hernández-Saavedra, 2012), and increased
434 observations of ceroid bodies (containing oxidized molecules) in tissues (Galimany et al.,
435 2008b; Lassudrie et al., 2014). Oxidation of the viral envelope could decrease virus viability,

436 but also may alter entry mechanisms into the host cells, as well as interfering with viral
437 replication (Lorizate and Kräusslich, 2011).

438 Cells of *Alexandrium* spp. have been reported to produce extracellular compounds with
439 allelopathic, hemolytic, ichtyotoxic, and oxidative properties, that can have deleterious effects
440 upon different target cells (Arzul et al., 1999; Flores et al., 2012; Lelong et al., 2011). Toxic
441 effects of extracellular compounds of *A. catenella* toward OsHV-1µVar particles could occur
442 through lysis or oxidation of the virus lipid-containing envelope and affect transmission
443 process directly in the water column. In fact, Paul-Pont et al. (2013) and Evans et al. (2014)
444 hypothesized that horizontal transmission of OsHV-1µVar would be promoted by planktonic
445 cells carrying the virus. Thus, by damaging the herpesvirus particle envelope, *A. catenella*
446 extracellular compounds may have decreased the viable viral particle load carried by algal
447 cells and consequently reduced the viable viral load entering the host oyster.

448 Another hypothesis to explain a lower transmission of OsHV-1µVar particles in oysters
449 exposed to *A. catenella*, is based upon the effects of *Alexandrium* spp. exposure upon bivalve
450 feeding activities. Indeed, filtration, clearance, and ingestion rates were reported to be reduced
451 during exposure to toxigenic algae (Cucci et al., 1985; Lassus et al., 1999; Shumway and
452 Cucci, 1987). Such effects could limit the introduction of viral particles into the organism. A
453 similar, hypothetic relationship between filtration rate and herpesvirus load has already been
454 proposed by Schikorski et al. (2011a).

455

456 Challenge of Specific Pathogen-Free (SPF) oysters by cohabitation with field-exposed oysters
457 led to a lower PST accumulation after exposure to *A. catenella*, compared with unchallenged
458 oysters. The difference in PST accumulation between challenged and unchallenged oysters

suggests that exposure to an environment with pathogens either: (i) affected the oyster filtration or ingestion rates or the digestive processes resulting in different PST load; or (ii) reduced the availability and / or the digestibility of the *A. catenella* cells.

The first hypothesis involves nutrition and digestion processes. Recent results of an experimental study suggested that food ingestion or absorption can be affected by OsHV-1 µVar and possible other pathogens associated with mortality events, as lower sterol contents were observed in oysters exposed to these pathogens (Tamayo et al., 2014). These authors also described oyster responses that mimicked food deprivation associated with the disease, as indicated by the utilization of proteins for energetic requirements following glycogen depletion. Depleted energetic reserves, coincident with mass mortality events in the field (Pernet et al., 2014), could be a consequence of stimulation of glycolysis and lipolysis by OsHV-1µVar infection, as suggested by a recent proteomic study (Corporeau et al., 2014). Additionally, ulcerative lesions of mantle and gill epithelium were observed in OsHV-1-infected oysters *C. gigas*, in Australia (Jenkins et al., 2013), possibly interfering with filtration and sorting involved in feeding. Altogether, findings from the present and recent studies could indicate alteration of feeding processes and digestive functions by viral or bacterial infections affecting *A. catenella* consumption by challenged oysters.

Finally, as mentioned previously, the interaction of *A. catenella* cells with micro-organisms carried by-field exposed oysters could have reduced availability or digestibility of the algal cells prior to filtration. In fact, bacteria can produce bio-active compounds with algicidal properties or exopolymers that may enhance algal cell sinking and degradation (see review of Doucette, 1995). In addition, when subjected to a stress, some dinoflagellates, including *Alexandrium* sp., are able to rapidly form temporary cysts, which can still be ingested by filter feeders; however, impenetrable cyst walls protect them from being digested and thus limit

toxin release (Hégaret et al., 2008; Laabir et al., 2007; Persson et al., 2006). Thus, a stressful effect of extra-cellular bacterial production also could have induced encystment of *A. catenella* cells, thereby decreasing their digestibility and resulting in lower toxin accumulation in the oyster digestive gland.

5 Conclusions

This study revealed that *A. catenella* can significantly impact the *C. gigas* – herpesvirus interaction. Additionally, the herpes virus infection, and / or putative associated microbiota, can change interactions between oysters and *A. catenella* and decrease oyster PST accumulation, demonstrating the complexity of such tripartite interaction.

The results show that *A. catenella* could partially inhibit transmission or proliferation of herpesvirus if a bloom occurs at the initial stage of the infection. The effect upon mortality events associated with OsHV-1µVar, however, remains to be investigated *in situ*, although these results suggest that an *A. catenella* bloom could delay the mortality outbreaks.

Further research focusing on direct interactions between toxic dinoflagellates and OsHV-1, as well as on oyster immune- and physiologically-related processes would be needed to better understand the mechanisms involved in this tripartite interaction and better project consequences upon oyster industry.

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760

Figure captions

Figure 1. Scheme of the experimental design. All oysters used were produced in hatchery in conditions naive from OsHV-1 and were designated as Specific Pathogen-Free (SPF). SPF and field-exposed oysters were sampled prior to exposure and cohabitation (T_0); field-exposed oysters, challenged and unchallenged oysters were sampled after 4 days of algal exposure and cohabitation (T_4).

Figure 2. Kaplan-Meier cumulative mortality curves in oyster spat according to field-exposure, challenged (cohabitation with field-exposed oysters) or unchallenged, and algal exposure (*Ht*: *Heterocapsa triquetra* or *Ac*: *Alexandrium catenella*). Significant differences between mortality curves are indicated by letters (Log-rank test adjusted with Holm-Bonferroni correction for multiple comparisons; $p<0.05$). At T_0, N =90 field-exposed oysters per algal treatment, 90 challenged oysters per algal treatment and 180 unchallenged oysters per algal treatment.

Figure 3. (A) OsHV-1 prevalence in challenged oyster spat, after 4 days exposure to the non-toxic *Heterocapsa triquetra* or the toxic *Alexandrium catenella*. * indicates a statistical difference between conditions (Chi-square test; $p<0.05$). N is indicated in each bar. (B) OsHV-1 infection intensity (considering only challenged oysters detected positive to OsHV-1) and weighted prevalence (considering all challenged oysters), quantified in the mantle after 4 days exposure to the non-toxic *H. triquetra* or the toxic *A. catenella*. * indicates a statistical difference (*t*-test; $p<0.05$). Mean ± SE. N is indicated in each bar.

781 Figure 4. Paralytic Shellfish Toxin (PST) accumulation after 4 days of exposure to
782 *Alexandrium catenella* in the digestive glands of live, unchallenged or challenged oyster spat.
783 * indicates a significant difference between conditions (Mann-Whitney test; $p<0.05$). Mean ±
784 SE. N=23 per condition.

Figure 1 color

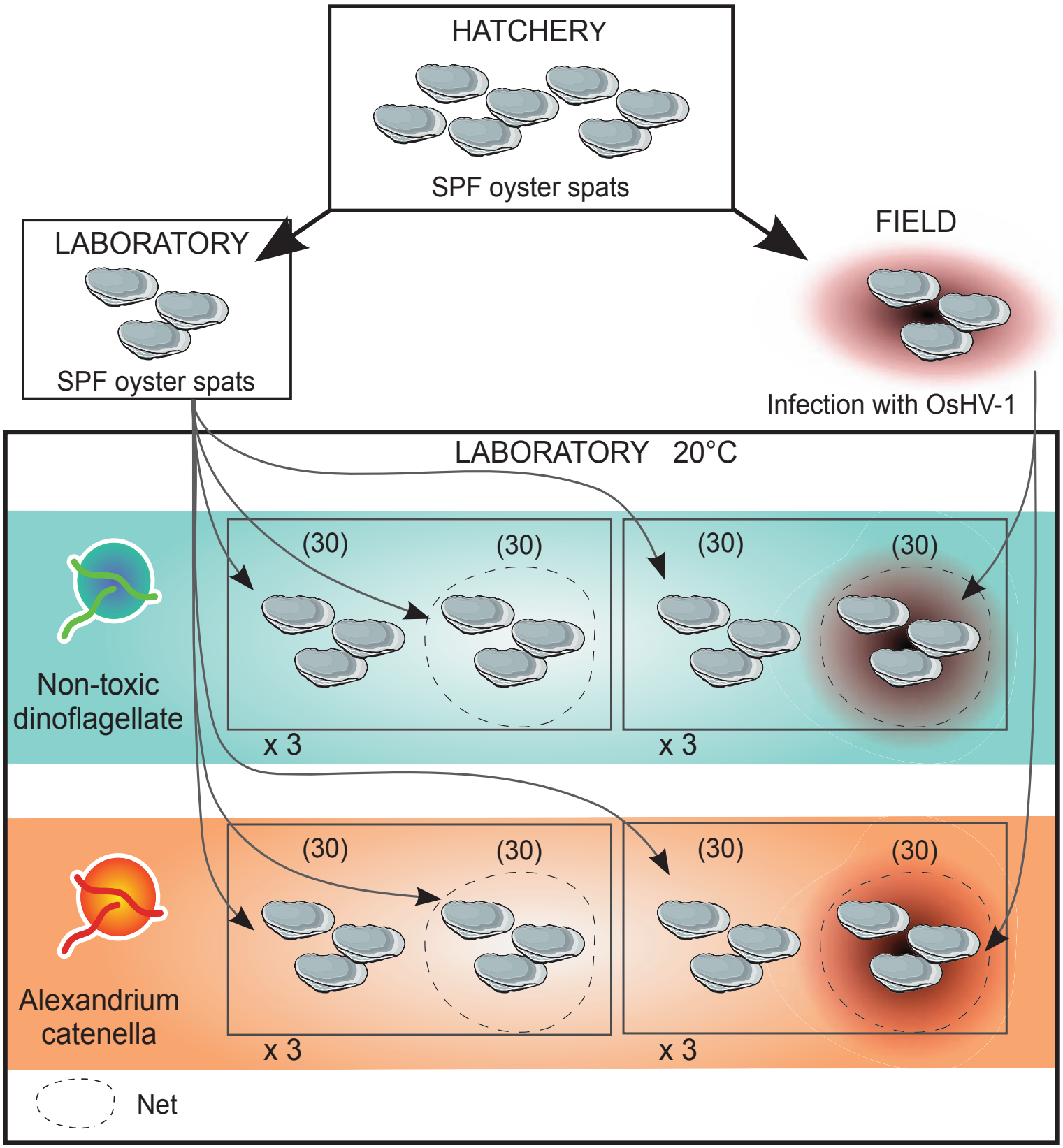


Figure 1 black & white

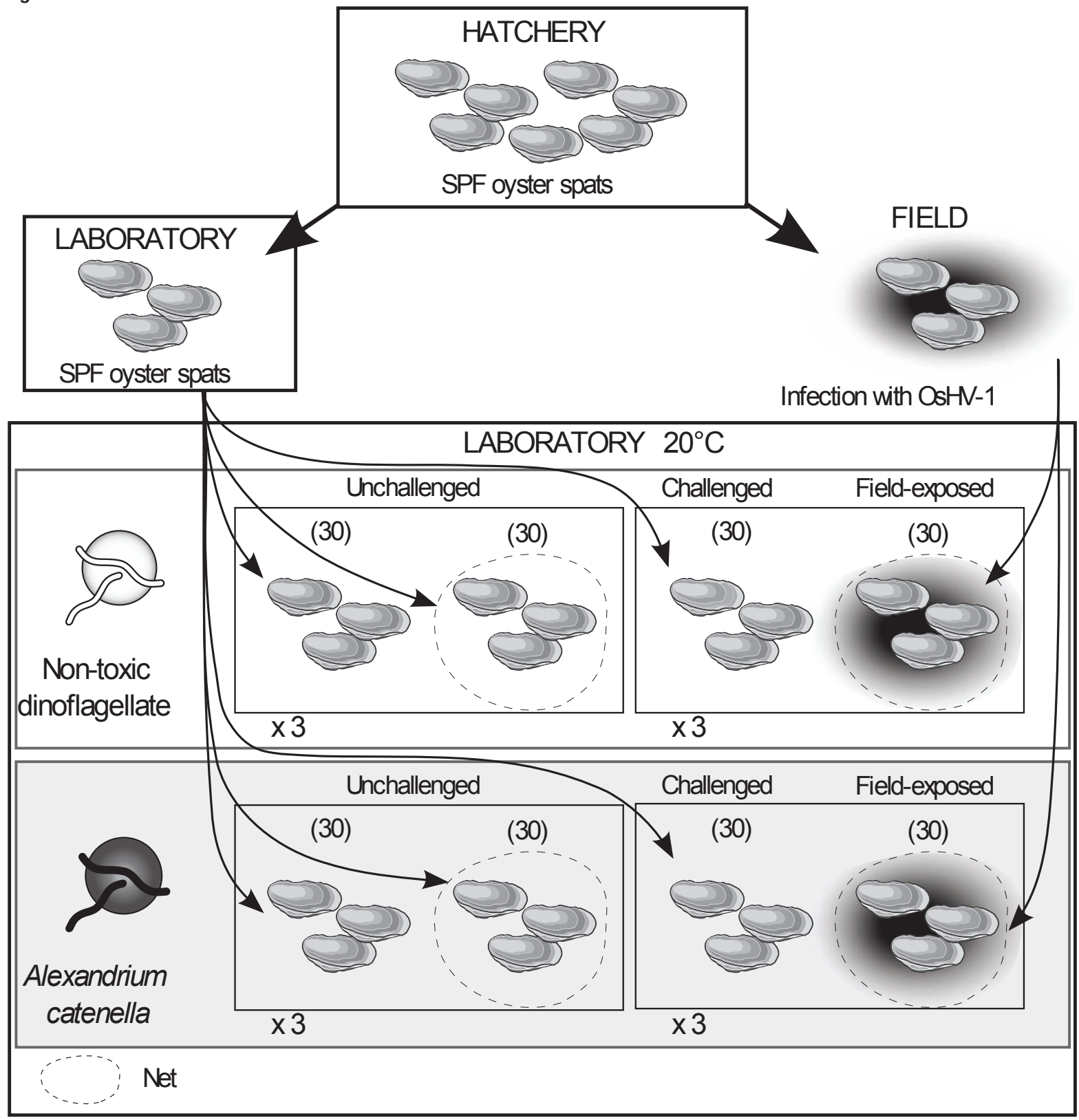


Figure 2

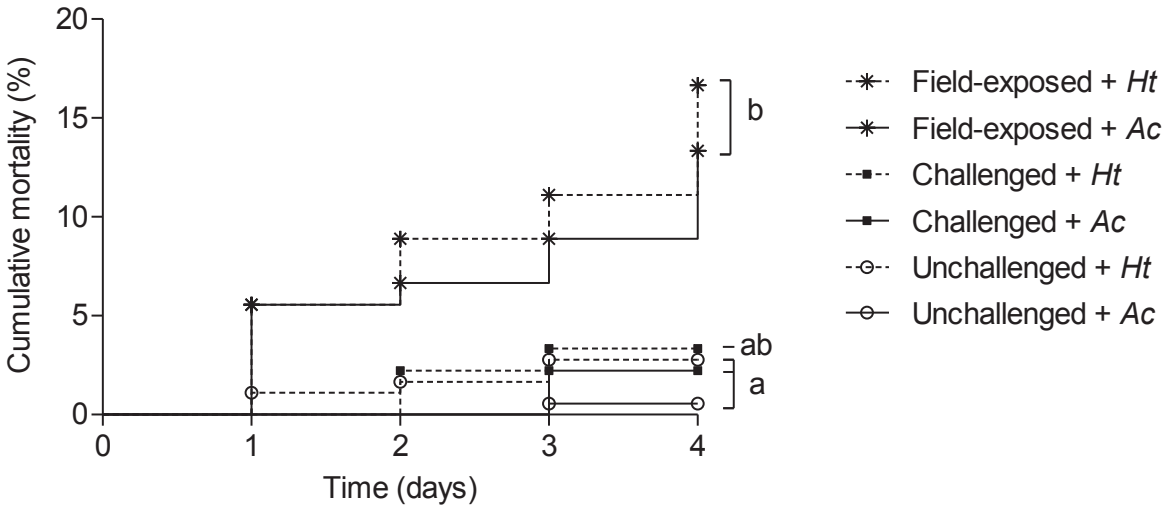

Cumulative mortality (%)
20
15
10
5
0
0
1
2
3
4
Time (days)
b
ab
a
Field-exposed + Ht
Field-exposed + Ac
Challenged + Ht
Challenged + Ac
Unchallenged + Ht
Unchallenged + Ac

Figure 3

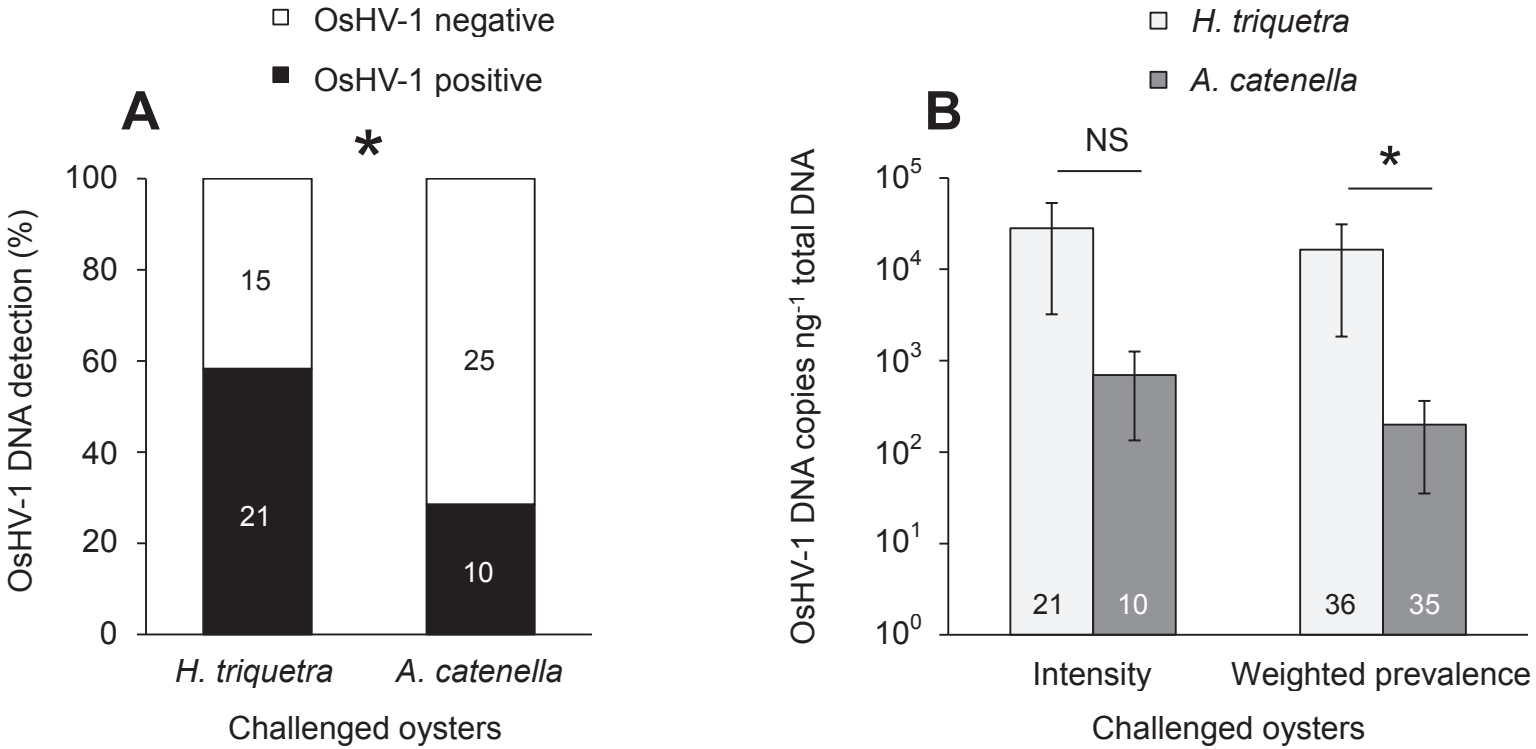
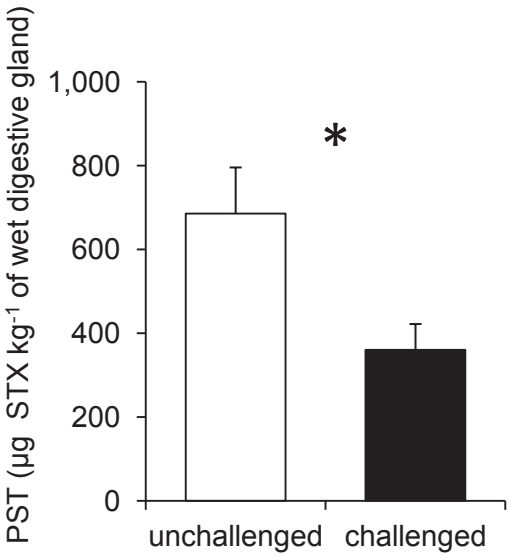

OsHV-1 negative
OsHV-1 positive
A
*
OsHV-1 DNA detection (%)
100
80
60
40
20
0
15
21
25
10
H. triquetra
A. catenella
Challenged oysters
H. triquetra
A. catenella
B
NS
*
OsHV-1 DNA copies ng-1 total DNA
10^5
10^4
10^3
10^2
10^1
10^0
21
10
36
35
Intensity
Weighted prevalence
Challenged oysters

Figure 4


PST (µg STX kg-1 of wet digestive gland)
1,000
800
600
400
200
0
*
unchallenged
challenged