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Inefficient immune response is associated with microbial permissiveness in juvenile oysters affected by mass mortalities on field

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Abstract :

Since 2008, juvenile *Crassostrea gigas* oysters have suffered from massive mortalities in European farming areas. This disease of complex etiology is still incompletely understood. Triggered by an elevated seawater temperature, it has been associated to infections by a herpes virus named OsHV-1 as well as pathogenic vibrios of the Splendidus clade. Ruling out the complexity of the disease, most of our current knowledge has been acquired in controlled experiments. Among the many unsolved questions, it is still ignored what role immunity plays in the capacity oysters have to survive an infectious episode. Here we show that juvenile oysters susceptible to the disease mount an inefficient immune response associated with microbial permissiveness and death. We found that, in contrast to resistant adult oysters having survived an earlier episode of mortality, susceptible juvenile oysters never exposed to infectious episodes died by more than 90% in a field experiment. Susceptible oysters were heavily colonized by OsHV-1 herpes virus as well as bacteria including vibrios potentially pathogenic for oysters, which proliferated in oyster flesh and body fluids during the mortality event. Nonetheless, susceptible oysters were found to sense microbes as indicated by an overexpression of immune receptors and immune signaling pathways. However, they did not express important immune effectors involved in antimicrobial immunity and apoptosis and showed repressed expression of genes involved in ROS and metal homeostasis. This contrasted with resistant oysters, which expressed those important effectors, controlled bacterial and viral colonization and showed 100% survival to the mortality event. Altogether, our results demonstrate that the immune response mounted by susceptible oysters lacks some important immune functions and fails in controlling microbial proliferation. This study opens the way to more holistic studies on the “mass mortality syndrome”, which are now required to decipher the sequence of events leading to oyster mortalities and determine the relative weight of pathogens, oyster

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genetics and oyster-associated microbiota in the disease.

Highlights

► Juvenile oysters susceptible to the disease mount an inefficient immune response associated with microbial permissiveness and death. ► Susceptible oysters were heavily colonized by OsHV-1 herpes virus as well as bacteria including vibrios potentially pathogenic for oysters. ► Adult oysters resistant to the disease expresses important immune effectors, controlled bacterial and viral colonization and survived to the mortality event.

Keywords : Host pathogen interaction, Innate immunity, Invertebrate, Mollusk, *In situ* mortality, Total bacteria, *Crassostrea gigas*

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57 ## Introduction

58 Over the past twenty years, recurrent mortality outbreaks have been recorded in the
59 production of *Crassostrea gigas* oysters [1, 2]. Since 2008, mortalities of high intensity and
60 wide geographic distribution have massively affected juvenile stages [3-9]. It is recognized
61 that this mortality syndrome refers to a multifactorial disease highly dependent on
62 temperature which is the main environmental stressor triggering the disease [4, 10-13].

63 This disease of complex etiology has motivated a broad number of studies and results
64 suggested that a combination of etiological agents, which include a virus and bacteria, are
65 responsible for the disease [3, 4, 14-16]. Among them, the most frequently incriminated are a
66 herpesviruses (OsHV-1 μVar) and pathogenic populations of vibrios of the Splendidus clade
67 [4, 16-20]. Recent works have demonstrated that OsHV-1 load correlated to oyster mortality
68 in the wild [3, 16, 21, 22]. However, the relative role of the diverse pathogenic agents in the
69 disease development, their interaction patterns and their dynamics during pathogenesis in
70 natural environment are largely unknown, which makes the disease difficult to understand,
71 predict and control. Beside an obvious role for pathogens, the current literature shows that the
72 severity of the disease is largely depended on host genetics and phenotypic plasticity. On the
73 one hand, Azema *et al.* showed that resistance to the disease is highly dependent on the oyster
74 genetics (from 0 to 100% of mortality in controlled conditions) and is a high heritable trait
75 [23]. On the other hand, a series of environmental and developmental factors were shown to
76 affect disease expression over oyster lifespan. These include oyster age, energetic reserves,
77 food quality and farming practices [11, 12, 24] . Both controlled laboratory experiments and
78 field experiments in which oysters were exposed to OsHV-1 showed that juvenile oysters
79 were more susceptible to the disease than adults [25, 26]. Consistently, oyster immune
80 capabilities were shown to change according to oyster developmental stages, abiotic stressors
81 (salinity, temperature and air exposure) and pathogens encountered (viruses and bacteria)
82 [27]. Particularly, experimental infections showed that juvenile and adult oysters mount
83 distinct immune responses against viral and bacterial pathogens associated to the disease [25,
84 28]. This immune plasticity is probably underpinned by the diversity of immune genes, which
85 have been the subject of massive expansion in *C. gigas* [27], but whose patterns of expression
86 remain poorly characterized.
87 For this reason, understanding the susceptibility of juveniles to mass mortalities requires to
88 conduct field studies that take in account the oyster immune response to abiotic factors and
89 natural pathobiome. A few field studies have been already conducted, but it is still unknown
90 whether the susceptibility of juvenile oysters to mass mortalities is due to an immature
91 immune system which would imperfectly sense pathogens or an incapacity to mount an
92 appropriate immune response [21, 29]. In other words, understanding the role of immunity in
93 the capacity of oysters to resist to the disease remains an important question to address to get
94 deeper insight into this complex disease.
95 With that objective, we conducted here a field study in which specific pathogen-free juvenile
96 oysters (8 months old), susceptible to the disease, were immersed in an oyster farms of the

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97 Thau lagoon (south of France) during an episode of mass mortality. Adult oysters (18 months
98 old and having survived a previous infectious episode) were used as a resistant control. The
99 immune response of the susceptible juvenile oysters and their colonization by microbes
100 including potential pathogens were monitored at four time points before and during the
101 infectious episode and compared to that of the resistant oysters maintained in the same
102 environment. Results showed that the susceptible oysters, which died by more than 90%, were
103 readily colonized by OsHV-1 and bacteria including vibrios of the Splendidus clade during
104 the infectious episode, as opposed to the resistant ones which maintained a stable bacterial
105 load, controlled pathogens and survived. Transcriptome analyses revealed that the susceptible
106 juvenile oysters mounted an inefficient immune response which differed from the efficient
107 immune status of the resistant oysters. Altogether, our results show that the susceptibility of
108 juvenile oysters is related to inefficient immune responses leading to microbial
109 permissiveness and death.
110
111
112 **Materials and Methods**
113
114 **Oyster sampling during *in situ* mortality**
115 Two different cohorts of *Crassostrea gigas oysters* were used for *in situ* experimentation.
116 Firstly, 18 months old oyster were produced in June 2008 at the Ifremer oyster hatchery in La
117 Tremblade (Charente Maritime, France). They were deployed in Thau lagoon in March 2009
118 in a site impacted by oyster mortality (latitude: 43.379087; longitude: 3.571483). Secondly, 8
119 months old oysters were produced in August 2009 at the Ifremer oyster hatchery in La
120 Tremblade (Charente Maritime, France). Genitors used to produce the two oyster cohorts
121 were collected from the same location (La Tremblade, France). They were deployed the 12
122 March 2010 in the same site as the 18 months old oysters. In Thau lagoon, 8 months old
123 oysters were collected at four dates (April 6, 12, 26 and 29, shell length 15±3 mm) and 18
124 months old oysters were collected at one date (April 29, shell length 62±9 mm). A
125 temperature monitoring was performed and mortalities were recorded. For each date and for
126 each oyster cohort, oysters were sampled (whole oyster flesh, 4 pools of 7 oysters) and snap-
127 frozen with liquid nitrogen and stored at -80°C until RNA and DNA extractions (for gene
128 expression analysis and pathogen quantification), intravalvular fluid was collected from other
129 oysters for cytometry analysis (10 individual oysters per condition, 100-400 µl per individual
130 fixed in 2% formalin (Sigma-Aldrich)), and six other oysters were collected to determine

131 abundances of culturable bacteria. In addition, environmental seawater was collected and
132 fixed in 2% formalin (Sigma-Aldrich) at each date during mortality (50ml) for cytometry
133 analysis, and sea water temperature was recorded every day (autonomous CTD
134 multiparameter recorders, NKE Instrumentation).
135

136 **RNA extraction and cDNA synthesis**

137 RNA extraction was performed following the TRIzol Reagent manual according to
138 manufacturer's instructions (Invitrogen) from frozen oyster powder (Retsch, Mixer Mill
139 MM400 with liquid nitrogen). Frozen oyster powder (20 mg) was homogenized in 1 ml of
140 Trizol by vortexing between 1-2h at 4°C. Prior to extraction, insoluble materials were
141 removed by centrifugation at 12000xg for 10min at 4°C. Next, RNA samples were treated
142 with 5 units of DNase I (Invitrogen) to eliminate DNA contamination, followed by heat
143 inactivation (10min at 65°C). Finally, RNA was precipitated with 100% isopropyl alcohol and
144 3 M Na-acetate, washed with 75% cold ethanol and dissolved in 50 µl of RNase-free water.
145 Quantification and integrity of total RNA was checked using a *NanoDrop* spectrophotometer
146 (Thermo Fisher Scientific) and 1.5% agarose gel electrophoresis, respectively. Total RNA (3
147 µg) was reverse transcribed in 20 µl using the Moloney Murine Leukemia Virus Reverse
148 Transcriptase (MMLV-RT) according to manufacturer's instructions (Invitrogen).
149

150 **DNA extraction**

151 For the genomic DNA (gDNA) extractions, 20 mg of frozen oyster powder was homogenized
152 in 500 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS and 0.1
153 mg/ml proteinase K, pH 8) for 4h at 50°C, followed by phenol/chloroform extraction and
154 precipitation with 100% ethanol for 2h at -80°C. gDNA was spooled with a pipette tip and
155 washed in tubes containing 75% ethanol, vacuum-dried, dissolved in DNase-free water and
156 treated with RNAse (Invitrogen) 1 mg/ml for 30 min at 37°C. A second precipitation was
157 performed with 100% isopropyl alcohol and 3 M Na-acetate and the gDNA pellet was
158 resuspended in DNase-free water. Quantification and integrity of gDNA was checked using a
159 *NanoDrop* spectrophotometer (Thermo Fisher Scientific) and 0.8% agarose gel
160 electrophoresis, respectively.
161

162 **Gene expression and pathogens quantification by quantitative PCR**

163 qPCR analysis were performed for two objectives: (i) for assessment of the relative
164 expressions of 88 genes of interest of the oyster from oyster cDNA, (ii) and for assessment of

165 the relative abundance of two pathogens (OsHV-1 and *Vibrio splendidus*) from oyster gDNA.
166 qPCR assays were carried out on the Light-Cycler 480 System (Roche Diagnostics GmbH).
167 The 5µl-volume reaction consisted of 1X Light-Cycler 480 master mix, 0.5µM of each primer
168 (Eurogentec) and 1µl of cDNA diluted at 1/8 in sterile ultra-pure water for gene expression
169 analyses, and 1 µl of gADN diluted at 20ng/µl in sterile ultra-pure water for pathogen
170 quantifications. qPCR assays were performed in triplicate, and primer pair efficiencies (E)
171 were calculated by five serial dilutions of pooled cDNA or gDNA ranging from 1/2 to 1/64 in
172 sterile ultra-pure water, in duplicate with each primer pair. Primer pair efficiencies were
173 calculated from the given slopes in LightCycler software according to the equation: E = 10[–
174 1/slope]. List of primers used to amplify the 88 immune related genes and the three reference
175 genes and primers used to amplify the two pathogens [30, 31] and the reference gene are
176 presented in **Supplementary table 1**. qPCR assays were submitted to an initial denaturation
177 step of 15 min at 95°C followed by an amplification of the target cDNA (35 cycles of
178 denaturation at 95°C for 10s, annealing at 57°C for 20s and extension time at 72°C for 25s)
179 and fluorescence detection. Relative expression of immune relative genes was calculated
180 using the $2^{-\Delta\Delta Ct}$ method [32], using the mean of the measured threshold cycle (Ct) values of
181 three constitutively expressed genes (*Cg-EF1* [GenBank AB122066], *Cg-RPL40* [GenBank
182 FP004478] and *Cg-RPS6* [GenBank HS119070]) to normalize the measured Ct values of
183 target genes. Moreover, relative abundance of the two pathogens was obtained with the same
184 method but using a single copy encoded gene (*C. gigas* bactericidal permeability-increasing
185 protein, *Cg-bpi*) as reference [GenBank: AY165040].

186

187 **Total Bacteria quantification**

188 Total bacterial cells were enumerated by flow cytometry from intravalvular fluids of 10
189 individual oysters per condition and from environmental seawater (previously fixed in
190 formalin 2%, Sigma-Aldrich) using SYBR Green I (Molecular Probes) according to the
191 protocol described by [33], modified by [34]. Samples were analyzed using a FACS Calibur
192 flow cytometer (from 20µl per sample, Becton Dickinson) equipped with an air-cooled argon
193 laser (488 nm, 15 mW). Stained bacterial cells, excited at 488 nm, were enumerated according
194 to their right angle light scatter (RALS) and green fluorescence (FL1) and measured using a
195 530/30 nm filter. Fluorescent beads (1-2 µm, Polysciences) were systematically added to each
196 sample to standardize the flow cytometer settings and true count beads (Becton Dickinson)
197 were added to determine the volume analyzed. Bacterial cells tend to cluster into two distinct
198 fractions based on differences in individual cell fluorescence (related to their nucleic acid

199 content) and in the side and forward light scatter signal (related to their cell size). These
200 fractions are defined as HNA cells (high nucleic acid content) and LNA cells (low nucleic
201 acid content), respectively [35, 36]. List-mode files were analyzed using BD Cell quest Pro
202 software (Becton Dickinson).
203 Additionally, abundances of culturable bacteria in whole oyster flesh were determined by
204 CFU counting. Oysters were sacrificed and ground in sterile seawater (10 mL/g of wet tissue).
205 The total culturable bacteria flora was quantified (CFU/mg of tissue) from six individual
206 oysters using serial dilutions on Marine agar (Becton Dickinson, Difco).
207

208 **Statistical analysis**

209 To get a global vision during oyster mortality of the modulated oyster genes according to *in*
210 *situ* conditions, qPCR data of all differentially expressed genes (one-way ANOVA, $p<0.05$,
211 Satistica software version 7.1) were analyzed by a hierarchical clustering with Multiple Array
212 Viewer software (version 4.6.2, http://www.tm4.org/mev/) using average linkage clustering
213 with Spearman rank correlation as the default distance metric.
214 Differences in microbial load between susceptible and resistant oysters at each date during
215 mortality (pathogens, total bacteria in intravalvular fluids, and culturable bacteria) were
216 determined by Mann–Whitney U test ($p<0.05$, GraphPad Prism 6).
217

218 **Results and discussions**

219

220 **Oyster mortalities on field**

221 In order to study oysters with contrasted phenotypes to mortality events, we used two
222 cohorts of *Crassostrea gigas* oysters in field experiment. On the one hand, 8 months old
223 specific pathogen free oysters directly issued from hatchery and susceptible to the disease [10]
224 were immersed in a farm of the Thau lagoon, south of France a few days before a *mass*
225 *mortality event* started (March 12th, 2010). One the other hand, 18 months old oysters having
226 survived an infectious episode one year before in the same farm were used as resistant
227 controls. Mortalities were monitored on both cohorts at different time points and temperature
228 was measured all over the experiment. Mortalities started when seawater temperature
229 exceeded 16°C, which is considered the low thermic threshold for *in situ* mortality outbreak
230 [10, 11]. As expected from our experimental design, mortalities were observed only on 8
231 months old oysters exposed for the first time to an infectious environment. A sharp rise in
232 mortality was observed, which reached 90% in only two weeks (from 12th to 29th of April)

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233 (**Figure 1**). Conversely, 18 months old oysters having survived a previous infectious episode
234 were only marginally affected, as indicated by less than 5% cumulative mortality by the end
235 of the experiment (**Figure. 1**).

236 This is in line with previous observations showing that OsHV-1-associated mortalities
237 affect preferentially spat and juvenile stages [24, 25, 37]. It also agrees with a previous study
238 showing that one-year-old oysters from one same origin were highly resistant (7% mortality)
239 when naturally selected for resistance to the disease whereas they died massively when they
240 have never been exposed to the disease before [11]. This was attributed to the strong genetic
241 basis of the resistance to the disease [25, 28]. However, like in our present experiment, other
242 important life history traits often differentiate oyster cohorts. Indeed, here adults have been
243 maintained in the Thau lagoon for more than a year. Not only did they pass though the major
244 selective filter of the disease (brought by a similar infectious environment) one year before,
245 but they have been confronted to an environment that can have modified their physiology and
246 associated microbiota, and/or primed their immune system as recently shown by Lafont *et al.*
247 [38]. Additionally, potential trade-offs between immune function, growth and reproduction
248 investment could also influence the resistance to the disease. Thus, both genetic factors and
249 phenotypic plasticity could influence the surviving capacity of adult oysters in our
250 experiment, but we cannot evaluate the relative weight of these two factors in the resistance
251 from our data.
252

253 **Oysters susceptible to the disease are highly permissive to bacterial colonization during**
254 ***in situ* mortality outbreak**

255 To determine the changes in bacterial abundance occurring in oysters with contrasted
256 resistance, we compared total bacterial loads in the susceptible (S, 8 months old oysters) and
257 resistant (R, 18 months old oysters) oysters during the mortality event. Bacterial load was first
258 measured by flow cytometry in intravalvular fluids of oysters, a compartment at the host-
259 environment interface that is potentially altered during bivalve diseases [39]. In addition,
260 bacterial load was measured in seawater surrounding oysters: no significant change was
261 observed between the early (April 26^{th,} 2010) and late stages (April 29^{th,} 2010) of the mortality
262 event with ~6.10^6 bacteria/ml of seawater at both time points (**Figure 2A**). Resistant oysters
263 did not show any significant bacterial colonization of their intravalvular fluids as indicated by
264 a constant bacterial load (from 2.10^6 to 1.10^7 bacteria/ml), similar to that of seawater, all over
265 the mortality event. Conversely, susceptible oysters, showed a high intravalvular fluid
266 bacterial load, up to 3.10^8 bacteria/ml, reaching 15 to 19 times the load measured in resistant

267 oysters, both at early and late stages of the mortality outbreak (Mann–Whitney U test,
268 $p<0.05$). Those results showed that susceptible oysters are significantly more permissive to
269 bacterial colonization than resistant oysters. Similar results were observed when we quantified
270 culturable bacteria into oyster flesh. The bacterial load in resistant oysters remained constant
271 over time, whereas the bacterial load of susceptible oysters was always significantly higher
272 compared to resistant ones (2.10^6 to 10^7 CFU/g *versus* 1 to 2.10^5 CFU/g, Mann-Whitney test,
273 $p<0.05$) (Figure 2B). Previous studies have already shown that *Vibrio* colonization precedes
274 oyster death [22] and that the stability of oyster microbiota influences the resistance of
275 pathogens [40]. However, these results show for the first time an intricate link between oyster
276 health status and bacterial colonization of both oyster flesh and body fluids. This event is
277 characterized by an overall stability in resistant oysters as opposed to a massive colonization
278 in susceptible oysters. Investigating the kinetic of bacterial colonization in resistant and
279 susceptible oysters before the occurrence of the first mortalities should be performed in the
280 future to evaluate the importance of bacterial colonization in the overall pathogenic process.
281

282 **Susceptible but not resistant oysters are heavily colonized by pathogens**

283 As oyster mortality outbreaks have been associated to both a herpesvirus (OsHV-1 µvar) and
284 vibrios of the Splendidus clade [4, 11, 15, 19, 41], we quantified their respective load in
285 oyster flesh by relative quantitative PCR. For vibrios, we used *V. tasmaniensis*-specific
286 primers designed on the sequence of the LGP32 pathogenic strain (Splendidus clade), which
287 was isolated during an *in situ* oyster mortality event [17]. This vibrio strain was used in
288 experimental challenges of oysters in different studies [4, 42]. The *V. tasmaniensis* load was
289 found to increase in susceptible oysters before mortality started and reached a maximum when
290 mortality reached 90%, on April 29th, 2010 (**Figure 3A**) (Mann–Whitney U test, $p<0.05$). The
291 oyster colonization by pathogenic strains of vibrios before the mortality outbreak is in
292 agreement with previous work [19, 22]. Concomitantly, OsHV-1 colonized tissues of
293 susceptible oysters (**Figure 3B**). A gradual increase in viral load was indeed observed all over
294 the time course. This increase became significant on April 26th, 2010 when mortality started.
295 The concomitant colonization of oyster flesh by OsHV-1 and vibrios of the Splendidus clade
296 preceding oyster death has been reported in different environments (Atlantic and
297 Mediterranean) [11, 15] and seems to be the hallmark of the disease. Thus, together with
298 previous studies [11, 15, 22], our data confirm the hypothesis of a temperature-dependent
299 polymicrobial disease in which both an OsHV-1 virus and pathogenic populations of vibrios
300 cooperate to kill oysters. Additionally, our results indicated that adult oysters which survived

301 the disease outbreaks were not tolerant but indeed resistant to the main pathogens associated
302 to the disease, which failed to colonize their intravalvular fluids and tissues. In the future,
303 moving to a non-candidate approach for pathogen monitoring in resistant and susceptible
304 genetic backgrounds appears essential for a complete understanding of the disease. Indeed,
305 not only did we observe here major bacterial proliferation in susceptible oysters (**Figure 2**),
306 but also recent studies reported that the stability of oyster/microbiota associations influences
307 the resistance of oysters to stress or invasion by pathogens [40, 43, 44]. Thus, to clearly
308 understand the role of microbiota in the pathogenesis, it will be important to analyze its
309 structure and dynamics throughout the infectious process.
310

311 **A set of immune genes specifically expressed in resistant oysters**

312 To unravel the difference in permissiveness between resistant and susceptible oysters, we
313 compared their immune status during the disease outbreak by analyzing the expression pattern
314 of genes previously described as key components of the oyster response to infectious
315 challenges [45, 46]. High-throughput RT-qPCR revealed that 54 immune-related genes out of
316 88 were differentially expressed (one-way ANOVA, $p<0.05$) between the two oyster cohorts
317 (resistant adults and susceptible juveniles) or between the different sampling times (i.e. before
318 or during mortalities) (**Figure 4**). On one dimension, hierarchical clustering of differentially
319 expressed genes separated samples into three clusters according to conditions (A-C): samples
320 from susceptible oysters fell into two clusters, before (cluster A) and during mortalities
321 (cluster C) whereas resistant oysters sampled during mortalities fell into a third cluster (cluster
322 B).

323 On the other dimension, genes also fell into 3 clusters according to expression (1-3). Genes
324 from cluster 2 were particularly interesting as they were expressed at higher ratios in resistant
325 oysters preferentially (cluster B) (**Figure 4**). Remarkably, cluster 2 is composed of genes
326 encoding pathogen recognition proteins (*lectins, and Complement C1q*) and immune effectors
327 including two antimicrobial peptides/proteins (*lysozyme, proline rich peptide*), a
328 *metalloproteinase inhibitor* and a *heat shock protein*, known to participate in oyster immune
329 response to bacterial infections [47-53]. Importantly, Cluster 2 also contains genes encoding
330 proteins involved in apoptosis (*caspase-3*) and autophagy (*beclin*), two processes that have
331 been described to play a key role in oyster response to viral and bacterial challenges [54, 55].
332 Since cluster 2 is specific to resistant oysters, which are neither colonized by OsHV-1 nor by
333 *V. tasmaniensis* (**Figure 3**), it is tempting to speculate that high expression of those
334 antibacterial and antiviral genes, either alone or in combination, plays an important role in the

335 control of pathogens and resistance to the disease. However, due to the design of the
336 experimental protocol, we do not have access to the dynamic of expression in resistant oysters
337 all along the mortality event (resistant oysters have been collected at the end of experiment
338 only). Thus, we cannot determine whether the high expression of genes from cluster 2 is due
339 to an induction of gene expression in resistant oysters as observed in experimental infection of
340 adult oysters by OsHV-1 [28] or whether it is due to a high constitutive level of expression as
341 observed in resistant oyster line compared to susceptible oyster line [56]. Indeed, constitutive
342 frontloading of stress tolerance genes was shown to maintain physiological resilience during
343 frequently encountered environmental stress in other species, particularly corals [57]. In the
344 future, investigating the dynamics of expression of genes from cluster 2 in field experiments
345 should help better understand the respective role of the antiviral and antibacterial immune
346 responses in controlling the pathogens associated to the syndrome.
347

348 **Oysters susceptible to the disease are able to sense pathogen colonization and trigger an**
349 **immune response**

350 Importantly, gene expression data revealed that susceptible oysters were able to actively
351 express immune genes when confronted to pathogens. Particularly, genes from cluster 1 were
352 highly induced in susceptible oysters over the mortality outbreak whereas resistant oysters
353 only poorly expressed those genes during mortalities (**Figure 4**). These genes encode notably
354 proteins involved in pathogen recognition (*galectin*) and immune signaling pathways (*Cg-*
355 *Myd88, NF-kappa-B inhibitor, MAP kinase-interacting serine/threonine-protein kinase,*
356 *interleukin 17*), and their overexpression has been associated with experimental challenges by
357 either bacteria or viruses [28, 58-61] as well as *in situ* infections of *C. gigas* oysters [62, 63].
358 These data clearly showed that susceptible oysters are able to detect pathogens and mount an
359 immune response.

360 However, a group of genes (cluster 3) showed decreased expression over disease development
361 in susceptible oysters (**Figure 4**). On the contrary, resistant oysters highly expressed cluster 3
362 genes during the mortality event. This contrast between resistant and susceptible oysters
363 suggests that repressed expression of cluster 3 genes is detrimental in susceptible oysters.
364 Genes from cluster 3 involved in the antioxidant system (*i.e. Super Oxyde Dismutase,*
365 *Glutathione S-transferase*) and metal homeostasis (*i.e. Metallothioneins*) have been
366 previously reported to be down regulated in oysters undergoing mass mortalities [60, 62] as
367 well as in experimental infections by OsHV-1 or pathogenic vibrios [28, 59]. ROS and metal
368 homeostasis were shown to play a major role in controlling vibrio infections, particularly in

369 the *C. gigas/V. tasmaniensis* LGP32 pathogenic interaction [42]. Disruption of those
370 important homeostatic processes might therefore have detrimental consequences on the host
371 and contribute to the massive increase in bacterial colonization observed in susceptible
372 oysters (**Figure 2**).
373 Altogether, our results showed that resistant and susceptible oysters, confronted to a mortality
374 outbreak in the field can be distinguished by distinct molecular immune signatures during
375 pathogenesis. Moreover, we showed that the although susceptible oysters juveniles sense
376 pathogens and signal an immune response, they do not express important functions related to
377 antimicrobial responses, apoptosis, ROS and metal homeostasis, thereby failing to control
378 pathogens and subsequent mortalities. The reason why pathogen sensing and immune
379 signaling does not result in pathogen control in juveniles remains to be explored both from the
380 host side looking closer at the genetic basis and the phenotypic plasticity of resistance and
381 from the pathogen side, as they could repress important host defense mechanisms for their
382 benefit.
383

384 **Conclusion**

385 By exposing oysters with fully contrasted resistance to a field mortality event and monitoring
386 simultaneously microbial load, specific pathogens and oyster immune status, we could
387 demonstrate that: (i) susceptible oysters sense pathogens and signal an immune response
388 during the mortality event, (ii) susceptible oysters do not express important antimicrobial and
389 apoptotic mechanisms and show repressed expression of genes involved in ROS and metal
390 homeaostasis, and (iii) this inefficient immune response enables major microbial colonization
391 by bacteria and viruses including species potentially pathogenic for oysters. Our data also
392 confirm the highly complex etiology of the juvenile oyster mortality syndrome which
393 associates both viruses and vibrios, and potentially other bacteria massively proliferating in
394 oyster flesh and intravalvular fluid during the mortality event. Understanding such a
395 mulfifactorial disease is no exception call upons the development of integrated and
396 multidisciplinary approaches [64] that will help deciphering the sequence of events leading to
397 oyster mortalities and determining the relative weight of pathogens, oyster (genetics and
398 phenotypic plasticity) and oyster-associated microbiota in disease expression and control.
399

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406

407 **Author contributions**

408 JdL, JME, YG, DDG, and EB designed the experiment and drafted the manuscript. FP, PLG,
409 and JdL designed and carried out the field experimentations. JdL and VL carried out the
410 qPCR analyses. AV performed sample conditioning and RNA extractions. FA, JLJ, and EJ-B
411 performed the quantification of culturable bacteria. PG performed the flow cytometry
412 analyses. All authors read and approved the final manuscript.

413

414 **Figure caption**

415 **Figure 1: Oyster mortalities monitoring during exposure in a culture area at Thau**
416 **lagoon (France).** Mortality were measured, at two dates before (6th and 12th of April) and two
417 dates after (26th and 29th of April) mortalities outbreak, on 8 months old oysters that were
418 exposed for the first time to an infectious environment (in red) and 18 months old oysters that
419 have already survived to a previous infectious episode (in bleu). Thau lagoon sea water
420 temperature is indicated by black dotted curve.

421

422 **Figure 2: Susceptible oysters are highly permissive to bacterial colonization during *in***
423 ***situ* mortality outbreak.** 8 months old oysters exposed for the first time to an infectious
424 environment (S) and 18 months old oysters that have already survived to a previous infectious
425 episode (R) were analyzed. **A:** Abundance of total bacteria in intravalvular fluid was
426 measured by flow cytometry at two dates (April $26^{th}$and 29th 2010) during mortality outbreak.
427 Abundance of total bacteria was measured in Thau lagoon sea water at the same dates (SW).
428 Results are the mean of six biological replicas (six individuals for oysters and six samples for
429 water), and significant differences of total bacteria abundance between conditions are
430 indicated by different lowercase letters (different letters indicate significant difference, a or b;
431 Mann–Whitney U test, $p<0.05$). **B:** Abundances of cultivable bacteria in whole oyster flesh
432 were determined by CFU count (on marine agar medium) at two dates (April $26^{th}$and 29th)
433 during mortality outbreak. Abundance of cultivable bacteria are determined from six
434 individual oysters per condition and significant differences between conditions are indicated

435 by different lowercase letters (different letters indicate significant difference, a, b or c; Mann-
436 Whitney U test, $p<0.05$).
437

438 **Figure 3: Pathogen detections before and during mortality outbreak.** *Vibrio tasmaniensis*
439 LGP32 (A) and OsHV-1 (B) were quantified by qPCR according to the $2^{-\Delta\Delta Ct}$ method [32]
440 using a single copy gene of oyster genome (*Cg-BPI*) to normalize Ct values of target genes
441 for *Vibrio tasmaniensis* LGP32 and for OsHV-1. Each relative value is the mean of tree
442 biological replicas (tree pools of 10 oysters per condition) and significant differences of
443 relative expressions between conditions are indicated by different lowercase letters (different
444 letters indicate significant difference, a, b or c) and were determined by the (Mann–Whitney
445 U test, $p<0.05$). 8 months old oysters exposed for the first time to an infectious environment
446 (S) and 18 months old oysters that have already survived to a previous infectious episode (R)
447 were analyzed.
448

449 **Figure 4. Gene expression of 54 immune related genes differentially expressed during**
450 **juvenile mortality.** Hierarchical clustering of the relative expression levels of 54 immune
451 related genes differentially expressed (one-way ANOVA, $p<0.05$) during mortality outbreak
452 was constructed with Multiple Array Viewer software using average linkage clustering with
453 Pearson Correlation as the default distance metric. Each cell in the matrix corresponds to the
454 expression level of one gene in one sample. The intensity of the color from green to red
455 indicates the magnitude of differential expression. Relative expressions were calculated
456 according the $2^{-\Delta\Delta Ct}$ method [32], from four groups of ten oysters per condition. The
457 dendrogram at the top of the figure indicate relationship among samples and revealed a
458 distinction between the tested conditions. The dendrogram at the left of the figure indicate a
459 relationship among the relative expression levels of the selected genes. Selected genes, listed
460 at the right of the figure.
461

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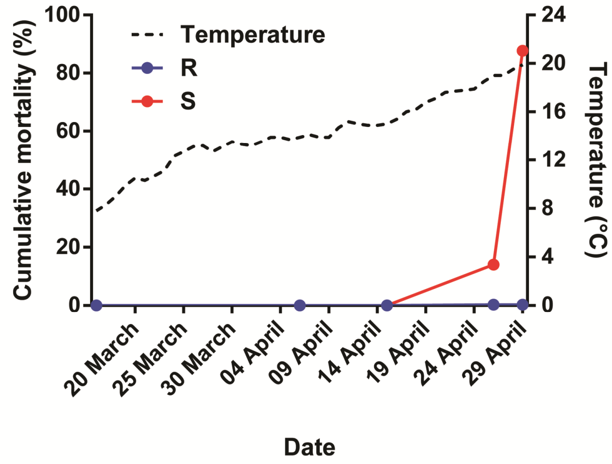

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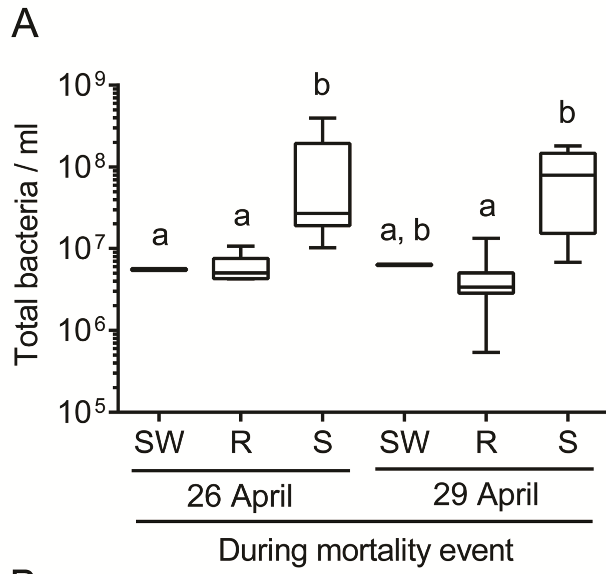

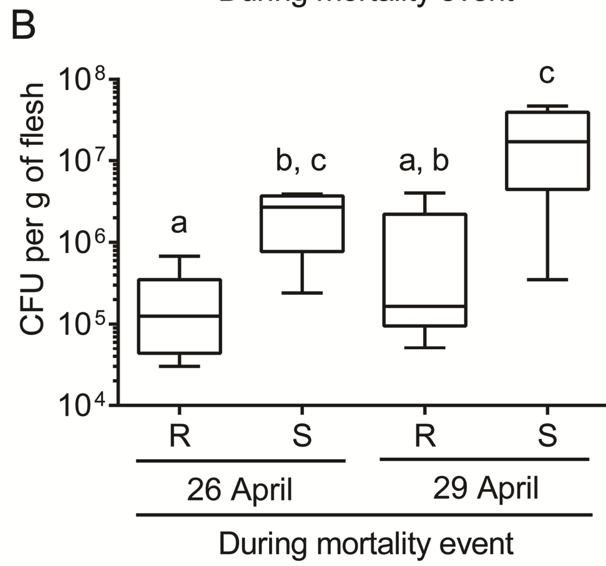

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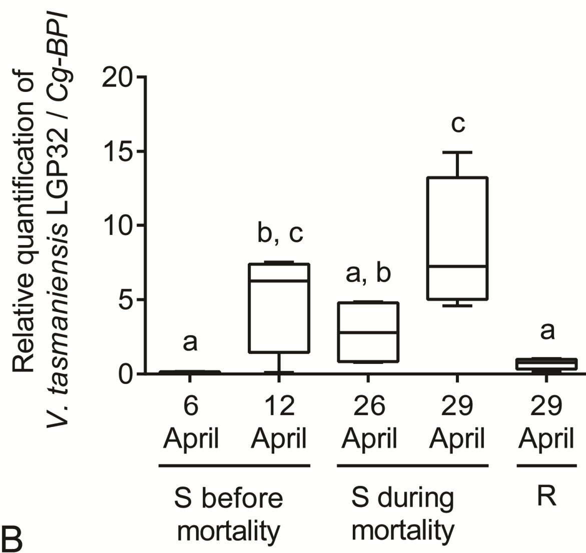

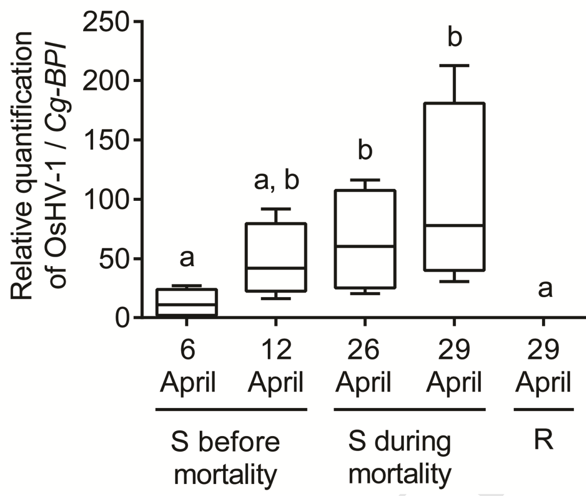

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Before mortality
During mortality
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0.0
1.0686133
2.0135233
-0.20817864
0.39591068
1.0
-0.15920138
0.4203993
1.0
A
B
C
1
2
3
Coadhesin
NF-kappa-B inhibitor epsilon
MAP kinase-interacting serine/threonine-protein kinase 1
Interleukin 17-like protein precursor
Myeloid differentiation primary response protein MyD88-like
Uncharacterized protein LOC105332551
Angiopoietin-1 receptor
N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase
Heat shock protein 70 B2
Alpha-crystallin B chain
No hit 76
Acyl-CoA desaturase
Neural-cadherin-like
Heat shock protein 70
uncharacterized protein LOC105321376
Galectin-8
Lysozyme
Proline rich protein
Short-chain collagen C4-like
Angiopoietin-1 receptor
Metalloproteinase inhibitor 2-like precursor
Histone-binding protein RBBP7-like
Hemagglutinin/amebocyte aggregation factor
No hit 302
Caspase-3-like
Fizzy-related-like protein
Mitogen-activated protein kinase kinase kinase 7
Calcium and integrin-binding protein 1-like
C-type lectin 2 like protein
Beclin-1
USP-like protein isoform 2
D-galactoside-specific lectin-like
Complement C1q-like protein 3
Hypothetical protein CGI_10001395
Tetratricopeptide repeat protein GNN
Ras-related protein Rab-35
Metallothionein
Ras-related protein Rap-1b
Activin receptor type-2B
Alpha-crystallin B chain
Metallothionein
Collagen alpha-1(XII) chain
Glutathione S-transferase omega-1
Succinate dehydrogenase
Superoxide dismutase
No hit 383
Calcineurin B subunit
Membrane frizzled-related protein
Beta-hexosaminidase subunit beta
Cystatin-A2
No hit 81
Hypothetical protein CGI_10004692
Caspase-3
Metallothionein

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Highlights

- Juvenile oysters susceptible to the disease mount an inefficient immune response associated with microbial permissiveness and death
- Susceptible oysters were heavily colonized by OsHV-1 herpes virus as well as bacteria including vibrios potentially pathogenic for oysters
- Adult oysters resistant to the disease expresses important immune effectors, controlled bacterial and viral colonization and survived to the mortality event.

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