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## Immune priming and portal of entry effectors improve response to vibrio infection in a resistant population of the European abalone

Dubief Bruno <sup>1,\*</sup>, Nunes Flavia <sup>1,2</sup>, Basuyaux Olivier <sup>3</sup>, Paillard Christine <sup>1</sup>

<sup>1</sup> Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR6539, CNRS/UBO/IRD/Ifremer, Institut Universitaire Européen de la Mer, University of Brest (UBO), Université Européenne de Bretagne (UEB), Place Nicolas Copernic, 29280, Plouzané, France

<sup>2</sup> Ifremer Centre de Bretagne, DYNECO, Laboratoire d'Ecologie Benthique Côtière (LEBCO), 29280, Plouzané, France

<sup>3</sup> Synergie Mer et Littoral, Centre Expérimental ZAC de Blainville, 50560, Blainville-sur-mer, France

\* Corresponding author : Bruno Dubief, email address : [bruno.dubief@univ-brest.fr](mailto:bruno.dubief@univ-brest.fr)

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### Abstract :

Since 1997, populations of the European abalone *Haliotis tuberculata* suffer mass mortalities attributed to the bacterium *Vibrio harveyi*. These mortalities occur at the spawning season, when the abalone immune system is depressed, and when temperatures exceed 17 °C, leading to favorable conditions for *V. harveyi* proliferation. In order to identify mechanisms of disease resistance, experimental successive infections were carried out on two geographically distinct populations: one that has suffered recurrent mortalities (Saint-Malo) and one that has not been impacted by the disease (Molène). Furthermore, abalone surviving these two successive bacterial challenges and uninfected abalone were used for several post-infection analyses. The Saint-Malo population was found to be resistant to *V. harveyi* infection, with a survival rate of 95% compared to 51% for Molène. While *in vitro* quantification of phagocytosis by flow cytometry showed strong inhibition following the first infection, no inhibition of phagocytosis was observed following the second infection for Saint-Malo, suggesting an immune priming effect. Moreover, assays of phagocytosis of GFP-labelled *V. harveyi* performed two months post-infection show an inhibition of phagocytosis by extracellular products of *V. harveyi* for uninfected abalone, while no effect was observed for previously infected abalone from Saint-Malo, suggesting that the effects of immune priming may last upwards of two months. Detection of *V. harveyi* by qPCR showed that a significantly greater number of abalone from the susceptible population were positive for *V. harveyi* in the gills, indicating that portal of entry effectors may play a role in resistance to the disease. Collectively, these results suggest a potential synergistic effect of gills and haemolymph in the resistance of *H. tuberculata* against *V. harveyi* with an important involvement of the gills, the portal of entry of the bacteria.

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## Highlights

► Susceptibility to vibriosis differs between abalone from two distinct populations. ► Immune priming is observed in abalone following a first exposure to *V. harveyi*. ► The detection of *V. harveyi* is lower in the gills of resistant abalone. ► The growth rate of *V. harveyi* was lower in the serum of resistant abalone. ► Both portal of entry and haemolymph effectors may play a role in resistance.

**Keywords** : Immunity, Haemocyte, Abalone, Disease, Extracellular products, Immune priming, *Vibrio harveyi*, flow cytometry, Resistance, Phagocytosis, Bacterial growth, qPCR, Gill

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## 1. Introduction

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In the natural environment, the interaction between pathogens and their hosts has important evolutionary repercussions, influencing genetic diversity of both hosts and pathogens [1,2]. According to the Red Queen hypothesis, each partner of this couple is in constant antagonist coevolution where pathogens evolve new arms to colonize the host, who in turn develop new features to counteract them. In a stable environment, this arms-race can lead to a balance that prevents one taking advantage over the other. However, global change has the potential to disturb this power relationship. Rapid environmental change can favor pathogens that have shorter generation times than hosts, and thus may adapt to new conditions more quickly whereas the hosts are weakened by them. Among new stressors, global warming is an important factor implicated in the emergence of disease [3,4], with adverse consequences for biodiversity. Many pathogenic bacteria grow preferentially in warm seawater, which can lead to an increase in the prevalence of disease with increasing temperature [5,6]. Increasing temperatures can also be detrimental to the immune system of invertebrate hosts thereby facilitating infection by a pathogen. For example, temperature increase leads to a reduction in phagocytosis and phenoloxidase in *Haliotis diversicolor* infected with *Vibrio parahaemolyticus* [4]. Similarly, temperature increase leads to a reduction in phagocytosis and superoxide dismutase activity, while an increase in total haemocyte count is observed in the hard clam *Chamelea gallina* [7]. Rising global temperatures can therefore affect both pathogen and host, potentially generating more favorable conditions for disease.

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The onset of massive mortalities of the European abalone *Haliotis tuberculata* is a compelling example of the de-stabilizing effects of environmental change on host-pathogen interactions. Since 1997, recurrent abalone mortality events of 50 to 90% have been attributed to the bacterium *V. harveyi* [8]. Field observations and laboratory studies of the disease etiology point to increasing water temperatures as the main cause of the disease. The first known mortalities were reported between Le Trieux and Saint-Malo, where

58 summer temperatures are among the highest in northern Brittany, France [9]. Subsequent disease outbreaks  
59 were limited to areas where summer temperatures exceeded 17.5°C [10]. In support of these field  
60 observations, experimental infections showed that *V. harveyi* was only able to cause death by septicemia when  
61 water temperatures exceeded 17°C during the spawning period of *H. tuberculata* [11]. In addition to  
62 temperature, gonadal maturation and spawning of *H. tuberculata* were found to be linked with immune  
63 depression characterized by a decrease in phagocytosis and phenoloxydase activity leading to greater  
64 susceptibility to the disease [12]. Other external stressors have also been found to lead to immune depression  
65 rendering *H. tuberculata* susceptible to disease [13]. However, while the combined effects of higher  
66 temperature and gametogenesis are required to trigger an infection in *H. tuberculata*, below 18°C, *V. harveyi*  
67 did not cause mortality in mature abalones [11], showing that temperature remains a key factor for the  
68 existence of the disease.

69         Given the rapid increase of sea surface temperatures [14] and the threat that it represents regarding  
70 the evolution of host-pathogen interactions, understanding whether and how marine organisms can defend  
71 themselves is of great concern. In the case of *H. tuberculata* infected with *V. harveyi*, the abalone immune  
72 system is rapidly affected by the pathogen, as already in the early stages of disease, phagocytosis, the  
73 haemocyte density and the production of reactive oxygen species are negatively impacted [15]. Moreover, *V.*  
74 *harveyi* can inhibit phagocytosis by inactivating p38 MAP kinase [16], avoiding the host immune system. While  
75 *V. harveyi* appears well-equipped to attack its host, less is known about the potential ability of *H. tuberculata* to  
76 defend itself against *V. harveyi*, and whether resistance to the disease exists. A successive infection experiment  
77 conducted on farmed abalone that aimed to select resistant individuals and identify potential effectors of  
78 resistance found that survivors over-expressed several genes implicated in metabolic regulation [17]. Because  
79 coping with stress such as gametogenesis and bacterial infection has an energetic cost, resistance to disease  
80 may therefore, be associated with individual or populational differences in metabolism and/or energy  
81 allocation strategies.

82         In the natural environment, recurrent mortality can select for disease resistance. In the black abalone  
83 *Haliotis cracherodii*, resistance against a rickettsial disease was found in the population of San Nicolas Island,  
84 which was historically the most impacted by the disease [18]. With respect to *H. tuberculata*, contrasting  
85 mortalities have been observed in natural populations in France, raising the question of whether resistant

86 populations can be found in areas highly impacted by the disease. Identification of a population tolerant or  
87 resistant to the disease could allow resistance factors to be identified.

88 An additional feature of the invertebrate immune system that can lead to an improved response  
89 against a pathogen is immune priming. Immune priming is an adaptive response of the immune system which  
90 provides protection in a similar way to the immune memory of vertebrates, but via different biologic  
91 mechanisms [19]. Invertebrate immune priming has been observed to be either a specific recognition of a  
92 pathogen that then leads to a faster and more intensive immune response at a second exposure [19] or a  
93 sustained non-specific immune response following a first infection [20]. In certain species, the enhanced  
94 immunity stimulated by priming can be transmitted to the next generation, providing an important advantage  
95 to the host in the context of emergent diseases [21]. Immune priming, if present in *H. tuberculata*, could be an  
96 important defense against *V. harveyi*.

97 The main objectives of this study were (1) to examine the existence of a population of *H. tuberculata*  
98 resistant to *V. harveyi* infection; (2) to compare the immune responses of different natural populations during  
99 successive exposures to the pathogen, in order to explain differences in tolerance to the disease and (3) to  
100 investigate a potential immune priming response of *H. tuberculata* against *V. harveyi*.

101

## 102 2. Methods

### 103 2.1 Abalone and bacterial strains

104 In order to identify populations with different susceptibilities to the disease, abalones were sampled in  
105 an area that has been recurrently impacted by the disease (Saint-Malo) and in a non-impacted area (Molène).  
106 Individuals from the two abalone populations were supplied by local commercial fishermen in May 2014: Saint-  
107 Malo (weight  $84.5 \pm 14.3$  g; shell length  $83.1 \pm 7.6$  mm) and Molène (weight  $82.5 \pm 19.1$  g; shell length  $84.2 \pm$   
108  $4.4$  mm). Abalones from the two populations were kept separate, but under the same controlled conditions for  
109 approximately five months and fed with *Palmaria palmata* ad libitum. During this period, the two populations  
110 received the same circulating water and the temperature was controlled such that the two populations reached  
111 maturity at the same time and as close as possible to the first infection (1300 degree-days). Abalones were  
112 transferred to infection tanks two weeks before the start of experimentation (September 2014). The  
113 temperature was set to 17°C ten days before experimentation and then increased to 18°C four days before the

114 infection, in order to prevent uncontrolled bacterial proliferation prior to the experiment. Dead abalones were  
115 counted and removed twice daily.

116 The bacteria used for the challenge is a virulent strain of *Vibrio harveyi* (ORM4) isolated from diseased  
117 abalone in Normandy, France during an episode of massive mortalities in 1999 [8]. Bacteria were grown in  
118 Luria-Bertani Agar supplemented with salt (LBS) at a final concentration of 20g.l<sup>-1</sup> during 72 hours at 18°C. Prior  
119 to use in the experiment, bacteria were washed with filter-sterilized seawater (FSSW) and quantified by optical  
120 density measurements at 490 nm. For the post-infection analyses, a modified mother strain of ORM4 was used:  
121 a kanamycin resistant strain, tagged with green fluorescent protein (GFP) [22].

122

### 123 2.2 Bacterial challenge

124 Abalones were challenged by two successive infections by immersion separated by a period of four  
125 weeks including three weeks of rest. They were placed in 100 L tanks in a closed system, supplied with  
126 seawater maintained at 18°C with a summer photoperiod (16h day/8h night) for a number of 22 abalones per  
127 tank. For each of the two populations, two conditions were performed in triplicate: a control condition with no  
128 bacterial exposure and another condition exposed to 10<sup>4</sup> CFU.ml<sup>-1</sup> of *V. harveyi* during 24 h. After the 24 h  
129 exposure, water in all the tanks was renewed at 100%, with subsequent water renewals of 50% taking place  
130 each day for the remainder of the infection. A second infection was performed with the surviving abalones 28  
131 days after the first exposure, following the same protocol. During the infection periods, abalones were not fed,  
132 but feeding with *P. palmata* resumed during the three-week rest period between the two infections. Dead  
133 abalones were counted and removed twice daily. The second infection was followed for one week. After this  
134 period, all surviving infected abalones and uninfected (control) abalones were kept for an additional two  
135 months at 18°C with a summer photoperiod and fed with *P. palmata* for post-infection analyses.

136

### 137 2.3 Sampling of abalone hemolymph and tissues

138 In order to compare the immune response of the two populations at different time intervals of the  
139 disease and between the first and the second infection, live abalone were sampled at 1, 3 and 5 days after the  
140 first infection and at 0 (just before exposure), 1 and 3 days after the second infection. Particular attention was  
141 given to not sample moribund abalones. For the second infection, sampling at 1 day was only possible for the  
142 Saint-Malo population because mortality in the Molène population was too high, and the number of animals

143 remaining was insufficient for 3 sampling points. For each sampling time point, three abalones were taken from  
144 each replicate tank. Approximately 5 mL of hemolymph was collected from each animal with a sterile syringe.  
145 300 µl of hemolymph were used immediately for *in vitro* phagocytosis and total haemocyte count, 500 µl was  
146 frozen at -20°C for the detection of *V. harveyi* by qPCR. All animals were dissected to collect the gills which  
147 were frozen in liquid nitrogen immediately.

148

#### 149 2.4 *In vitro* phagocytosis index, viability and Total Haemocytes Count (THC)

150 Freshly collected hemolymph was used immediately for quantifying phagocytosis, total haemocyte  
151 count (THC) and viability by flow cytometry. Three technical replicates were run for each biological replicate.

152

##### 153 2.4.1 *Viability index and THC*

154 50 µl was diluted in 150 µl of AASH supplemented with 1% of Sybr Green fluorescent dye and 1% of  
155 propidium iodide fluorescent dye in a 96-well round-bottom plate. After 20 min incubation at room  
156 temperature, the plate was analyzed by flow cytometry. While Sybr Green binds to all nucleic acids present in  
157 the sample, and is measured by green fluorescence, propidium iodide can bind only to dead cells that have  
158 suffered loss in membrane integrity and is measured by red fluorescence. The THC value was obtained by the  
159 number of events showing a green fluorescence divided by the flow rate. The viability index was determined as  
160 the percentage of haemocytes which did not show red fluorescence.

161

##### 162 2.4.2 *Phagocytosis index using fluorescent beads*

163 50 µl of hemolymph was diluted in 100 µl of FSSW and distributed in a 96-well round-bottom plate.  
164 Haemocytes were allowed to adhere for 15 min, followed by the addition of 50 µl of 2.00 µm fluorescent beads  
165 diluted 1:100 in distilled water (Fluoresbrite YG Microspheres, Polysciences) [12]. After 1 h incubation at 18°C,  
166 the supernatant was removed and 50 µl of trypsin (2.5 mg.ml<sup>-1</sup>) in an anti-aggregating solution (AASH: 1.5%  
167 EDTA, 6.25 g L NaCl, in 0.1 M phosphate buffer, pH 7.4) was added to detach the cells from the bottom of the  
168 wells. After 10 min of shaking at the maximum speed on a Titramax 100 plate shaker (Heidolph), 150 µl of AASH  
169 was added and the plate was analyzed by flow cytometry with the Guava EasyCyte Plus (Merck Millepore).  
170 Beads were identified by their green fluorescence and the phagocytosis index was defined as the percentage of  
171 haemocytes phagocytosing three or more beads.

172

173 *2.4.3 Detection of V. harveyi in hemolymph and gills by real-time quantitative PCR*

174 DNA was extracted from 500 µl total hemolymph, and from 30 mg of gill tissue using the QIAamp DNA  
175 mini kit (QIAGEN) according to the manufacturer's protocol. Hemolymph samples were centrifuged at 10 000g  
176 for 10 min. Pellets containing bacteria and haemocytes were digested during 2 h with ATL buffer supplemented  
177 with 20 µl proteinase K. Frozen gills were ground with a MM400 mixer mill (RETSCH) and kept frozen using  
178 liquid nitrogen. After all subsequent steps of the standard protocol, the columns were eluted twice, first with  
179 150 µl of DNase free water, then with 50 µl. In order to quantify *V. harveyi* in hemolymph, a standard curve was  
180 obtained with 10-fold serial dilution in FSSW of *V. harveyi* bacterial culture, ranging from 10<sup>8</sup> to 0 CFU. In order  
181 to obtain a standard curve for gill tissue, bacterial culture was mixed with uninfected gill tissue homogenate in  
182 order to extract DNA under the same conditions as infected gill tissue. A 10-fold serial dilution of bacterial  
183 culture from 10<sup>7</sup> to 0 CFU were added to an uninfected abalone gill homogenate of 30 mg.ml<sup>-1</sup>. Bacteria  
184 concentration was estimated using a Malassez counting chamber under light microscopy.

185 The concentration of *V. harveyi* was quantified by qPCR using the LightCycler 480 Probes Master  
186 chemistry on a LightCycler 480 thermocycler (Roche). Amplification of the *V. harveyi tox-R* gene was done with  
187 the following specific primers: ToxR F1 CCA-CTG-CTG-AGA-CAA-AAG-CA and ToxR R1 GTG-ATT-CTG-CAG-GGT-  
188 TGG-TT. Fluorescent visualization of amplification was done using a *tox-R* probe dually labeled with the Texas  
189 Red 5' reporter dye and the BHQ-2 downstream 3' quencher dye: CAG-CCG-TCG-AAC-AAG-CAC-CG [23]. Each  
190 PCR reaction was run in triplicate, containing 5 µl of DNA, 600 nM of each primer, 200 nM of probes and 7.5 µl  
191 of master mix, for a final volume of 15 µl. Thermal cycling consisted of an initial pre-incubation step at 95°C for  
192 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing and extension at 58°C for 1 min and 30  
193 s, the fluorescence reading at each cycle at 72°C for 1 second. The thresholds were set using LightCycler 480  
194 software V 1.5 (Roche). The primer efficiency was determined by the slope of the standard curves using the  
195 equation  $E = 10^{(-1/\text{slope})}$ .

196

197 *2.5 Post-infection analyses*

198 Abalones surviving the experimental infections (from the Saint-Malo population) and uninfected  
199 (control) abalones (from both Saint-Malo and Molène) were used for several additional analyses. No survivors  
200 remained for the Molène population after two successive bacterial challenges. Additional analyses included: (1)

201 3D microscopy of intracellular uptake of *V. harveyi* by abalone haemocytes, (2) impact of extracellular products  
202 of *V. harveyi* on phagocytosis, and (3) the impact of abalone serum on bacterial growth. To minimize stress  
203 each individual was sampled for hemolymph only once.

204

#### 205 2.5.1 3D Microscopy of phagocytosis

206 Because the methods in flow cytometry used here cannot distinguish whether bacteria are adhered to  
207 the surface of a haemocyte or are internalized into the haemocyte, intracellular uptake of *V. harveyi* by abalone  
208 haemocytes was confirmed by 3-dimensional fluorescence microscopy for abalone from both Saint-Malo and  
209 Molène. Freshly collected hemolymph was diluted ten times in FSSW (100 $\mu$ l) and allowed to adhere for 15 min  
210 on a glass slide. The supernatant was removed and replaced by a GFP *V. harveyi* suspension [22] to obtain a  
211 50:1 bacteria to haemocyte ratio. After 1h of incubation at 18°C, the supernatant was removed and glass slides  
212 were washed twice with Phosphate Buffered Saline (PBS) pH 7.4 before being fixed for 10 min with 3.7%  
213 formalin in PBS. After two washing steps with PBS, glass slides were covered by 100  $\mu$ l of a dilution of  
214 methanolic stock solution of rhodamine-phalloidin R415 (Invitrogen) with PBS for 20 min, in order to label the  
215 actin of cytoskeleton, washed again in PBS, and covered with 100  $\mu$ l of 4', 6-diamidino-2'-phenylindole,  
216 dihydrochloride (DAPI) at 0.1  $\mu$ g.ml<sup>-1</sup> (Thermo Scientific) for 5 min, to label the nucleus. Finally, the slides were  
217 washed with PBS before observation. Slides were observed on an Axio Observer Z1 complemented by the 3D  
218 Vivatome module (Carl Zeiss AG). Lasers were used at  $\lambda_{ex}$  494nm  $\pm$  20 for GFP ( $\lambda_{em}$  436  $\pm$  40),  $\lambda_{ex}$  406 nm  $\pm$  15 for  
219 DAPI ( $\lambda_{em}$  457  $\pm$  50), and  $\lambda_{ex}$  575 nm  $\pm$  25 for rhodamine-phalloidin ( $\lambda_{em}$  628  $\pm$  40). In order to obtain a 3-  
220 dimensional image, series of 14 optical cross-sections of 0.8 $\mu$ m were collected and compiled. The images were  
221 processed with the AxioVision V 4.8 software (Carl Zeiss AG).

222

#### 223 2.5.2 Impact of extracellular products of *V. harveyi* on phagocytosis of GFP-labelled bacteria

224 Bacterial extracellular products (ECPs) were produced by the cellophane overlay method [24]. Luria  
225 Bertani agar plates were covered with sterile cellophane films, and then 2 ml of approximately 10<sup>9</sup> *V. harveyi*  
226 culture was transferred to the top of the cellophane and incubated at 18°C for 72h. The bacteria and their ECPs  
227 were recovered by successive rinsing with 4 ml of FSSW. The ECP/bacteria suspension was centrifuged at 10000  
228 g for 30 min, and the supernatant containing ECPs was recovered and filtered at 0.2  $\mu$ m. ECP concentration  
229 was measured by the Bradford method [25], with serum albumin as the standard. Phagocytosis of GFP-labelled

230 *V. harveyi* was measured in the presence of two concentrations of ECPs: 15 and 30  $\mu\text{g}\cdot\text{ml}^{-1}$ , and a positive  
231 control of FSSW containing no ECPs (0  $\mu\text{g}\cdot\text{ml}^{-1}$ ). Flow cytometry was performed on the Guava EasyCyte Plus  
232 (Merck Millepore). Phagocytosis values were represented by the mean of green fluorescence following the  
233 protocol described in Pichon et al. (2013), using 1 hour incubation.

234

### 235 2.5.3 Impact of abalone serum on bacteria growth

236 The ability of *V. harveyi* to grow in the serum of abalone from Molène and from Saint-Malo was tested  
237 using two strains: the virulent ORM4 and the non-virulent strain LMG 7890 [16]. For each abalone population, 1  
238 ml of hemolymph from 5 individuals was pooled, and then centrifuged at 200 g for 10 min in order to recover  
239 only the serum, followed by filter-sterilization at 0.2  $\mu\text{m}$ . Measurements of both ORM4 and LMG 7890 growth  
240 were done in 100-well flat-bottom plates with a computer-controlled incubator/reader/shaker, the Bioscreen C  
241 MBR. 150  $\mu\text{l}$  of serum and 50  $\mu\text{l}$  of bacteria suspension in LBS ( $4\cdot 10^4$  cells. $\text{ml}^{-1}$ ) were pipetted into each well. In  
242 order to have a stable control against which to compare bacterial growth in the serum of abalone from Molène  
243 and Saint-Malo, a positive control of growth was performed for both strains by adding 50  $\mu\text{l}$  of bacteria  
244 suspension with 150  $\mu\text{l}$  of LBS. A negative control was performed by adding 50  $\mu\text{l}$  of sterile LBS with 150  $\mu\text{l}$  of  
245 serum. Each condition was performed in triplicate. The plate was incubated at 18°C for 42 hours and the  
246 absorbance at 492 nm was measured at intervals of 30 min. The bacterial concentration was calculated with  
247 the following formula:  $6 \cdot 10^9 \times OD + 2 \cdot 10^8$  [22]. The maximum growth rate for each condition was obtained  
248 by calculating the slope of the bacterial exponential growth phase from a plot of the natural logarithm of  
249 bacterial abundance versus incubation time.

250

### 251 2.6 Statistical analysis

252 The survival rate of infected and uninfected abalone from Saint-Malo and Molène was computed with  
253 a Kaplan-Meier estimate followed by a log-rank test in the R “survival” package[27]. Phagocytosis and THC data  
254 were fitted on a linear mixed effects model with the factor tanks as random effect, followed by a pairwise  
255 comparison of the least-squares means between uninfected and infected treatments. The effects of ECP on  
256 phagocytosis for each population were estimated by a 2-way nested ANOVA. As the hemolymph of each  
257 individual was used to quantify phagocytosis under three concentrations of ECPs (conditions 0, 15 and 30  
258  $\mu\text{g}\cdot\text{ml}^{-1}$ ), individuals were considered as repeated factors. Then pairwise comparisons of the least-squares

259 means between ECP treatments were performed. Finally, a logistic regression model was used to investigate  
260 the link between the phagocytosis index and the probability of abalone to be positive for *V. harveyi*; and to  
261 evaluate if there were differences in the probability of being positive for *V. harveyi* for abalone originating from  
262 Saint-Malo or Molène. In all tests, at the significance threshold was set to  $\alpha = P < 0.05$ . All statistical analyses  
263 were performed using the software R (version 3.2.3)[28].

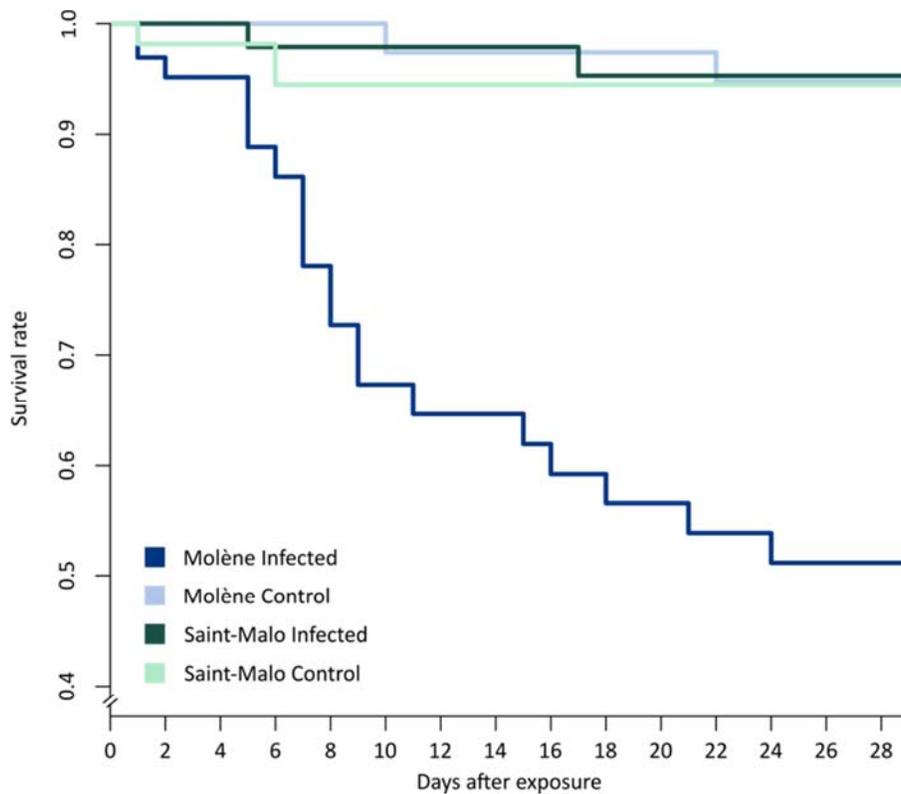
264

### 265 **3. Results**

266

#### 267 *3.1 Differential survival of abalone following V. harveyi infection in Saint-Malo and Molène*

268 The survival of abalones was measured in order to observe differences in resistance to infection with  
269 *V. harveyi* between the two populations. Indeed, during the first infection, the population of Molène suffered  
270 great mortalities whereas the population of Saint-Malo exhibited very little mortality. After the last observed  
271 mortality (24 days after the first exposure), the survival rate was 0.512 for Molène and 0.953 for Saint-Malo  
272 (Fig. 1). Survival for the Saint-Malo population was not significantly different from the uninfected controls  
273 ( $P=0.765$ ). The log-rank test showed a significant difference in survival between the two infected populations  
274 ( $P<0.001$ ). Survival was quantified only until 7 days after the second exposure, and by this time point no  
275 additional mortality was observed.



276

**Fig. 1** : Kaplan-Meier survival rate following the first exposure of the two populations to  $10^4$  bacteria/mL during 24 hours at  $18^\circ\text{C}$

277

278

### 3.2 THC and Viability during the successive infections

279

280

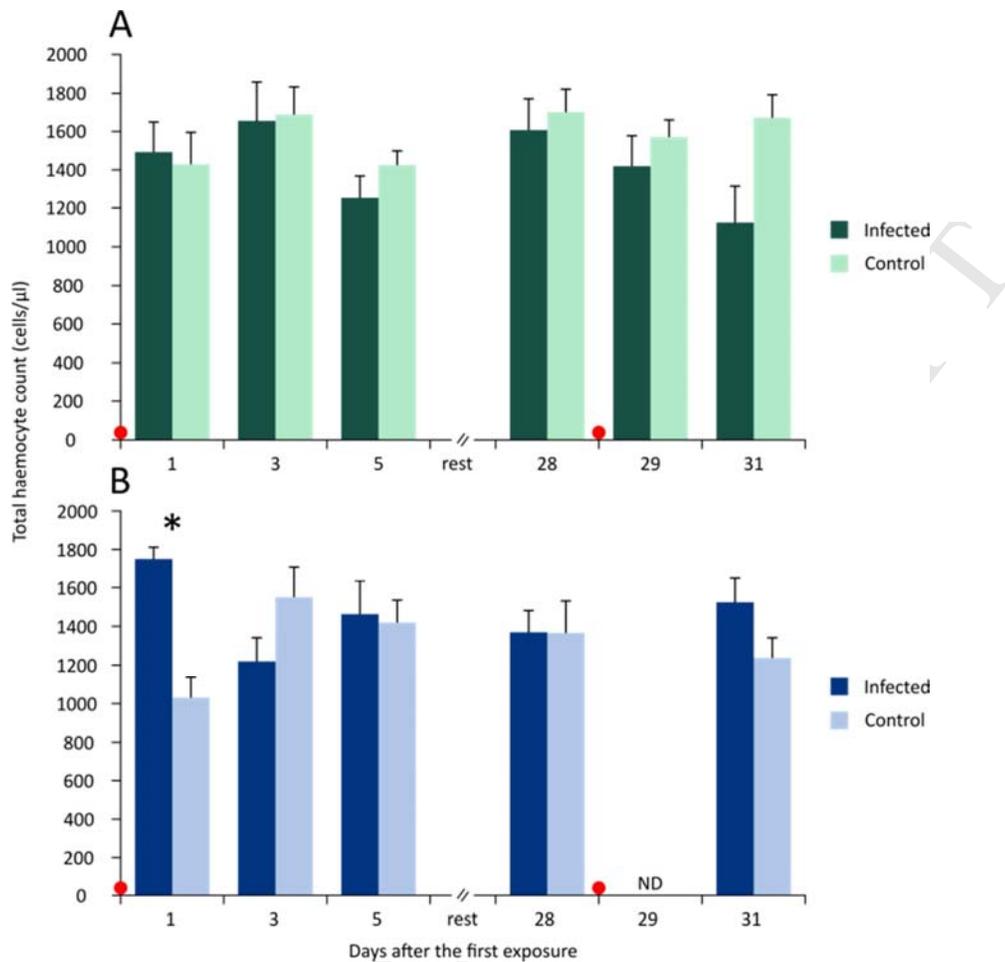
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Total haemocyte count (THC) and haemocyte viability were measured for individuals from Saint-Malo and Molène. Whereas no significant differences in THC were observed in abalones from Saint-Malo across all time points, abalones from Molène exhibited a significant increase in THC after 24h of exposure (Fig. 2). A slight decrease of THC was observed during the second infection for abalones from Saint-Malo but was not significant ( $P=0.0947$ ). No change was detected in the viability of haemocytes during the successive infections in both populations. The mean of haemocyte viability was  $98.44\pm 0.09\%$  across all individuals measured.



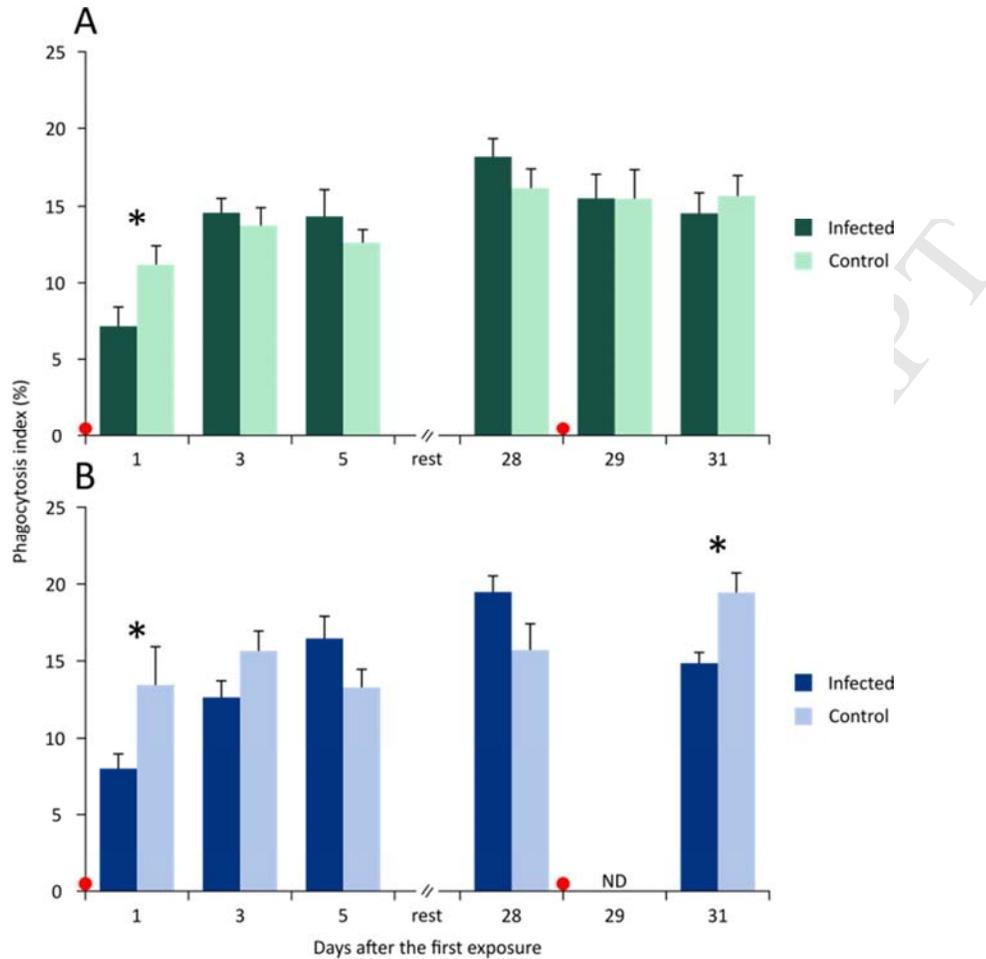
285

**Fig. 2:** Total haemocytes count (THC) during the two exposures of (A) Saint-Malo and (B) Molène. Red dots indicate the exposures. ND indicates that no data is available. \* indicates values that are significantly different from the control for a pairwise comparison of the least-squares means ( $p < 0.05$ ).

286

### 287 3.3 Phagocytosis index during the successive infections

288 A significant decrease in the phagocytosis index was observed 1 day after the first exposure to *V.*  
 289 *harveyi*, of 36% for Molène and 40% for Saint-Malo, relative to the uninfected abalone (Fig. 3). By 2 days, the  
 290 phagocytosis index of infected abalones recovered to the level of the uninfected controls and remained at the  
 291 level of the controls until 5 days post-infection. Just prior to the second exposure, no significant difference in  
 292 the phagocytosis index was observed between infected abalone and uninfected controls for the two  
 293 populations. During the second infection, no reduction in phagocytosis was observed in the infected abalones  
 294 from Saint-Malo and the level of phagocytosis was not significantly different than that of uninfected controls  
 295 until the end of the experiment. In contrast, abalone from Molène showed a significant decrease in  
 296 phagocytosis 5 days after the second exposure. (Fig. 3)



297

**Fig. 3:** Phagocytosis index based on micro-beads engulfment (percentage of haemocytes containing three or more fluorescent beads relative to total haemocytes) during two successive infections of abalone from (A) Saint-Malo and (B) Molène. Red dots indicate the timing of bacterial exposure. ND indicates that no data is available. \* indicates values that are significantly different from the control for a pairwise comparison of the least-squares means ( $p < 0.05$ ).

298

### 299 3.4 Detection of *V. harveyi* in hemolymph and gills by qPCR

300 The sensitivity threshold of qPCR was estimated to be  $7.5 \cdot 10^2$  bacteria/ml of hemolymph and  $2.5 \cdot 10^2$   
 301 bacteria/30 mg of tissue for gills. *V. harveyi* was detected in the gills of 20 individuals and in the hemolymph of  
 302 7 individuals (fig. 4). The concentration of bacteria was in the range of  $5.51 \cdot 10^2 \pm 57$  bacteria/30 mg of gills and  
 303  $6.4 \cdot 10^3 \pm 2.02 \cdot 10^3$  bacteria/ml of hemolymph. One individual showed  $1.8 \cdot 10^4$  bacteria/ml of hemolymph, which  
 304 explains the high standard deviation for this compartment (the average without this outlier individual was  
 305  $4.52 \cdot 10^3 \pm 7.23 \cdot 10^2$  bacteria/ml of hemolymph). As the concentration of *V. harveyi* was near the detection  
 306 threshold for most samples from which *V. harveyi* was detected, the detection results were treated as positive  
 307 or negative for the presence of the bacterium. A binomial logistic regression was used to treat these results.

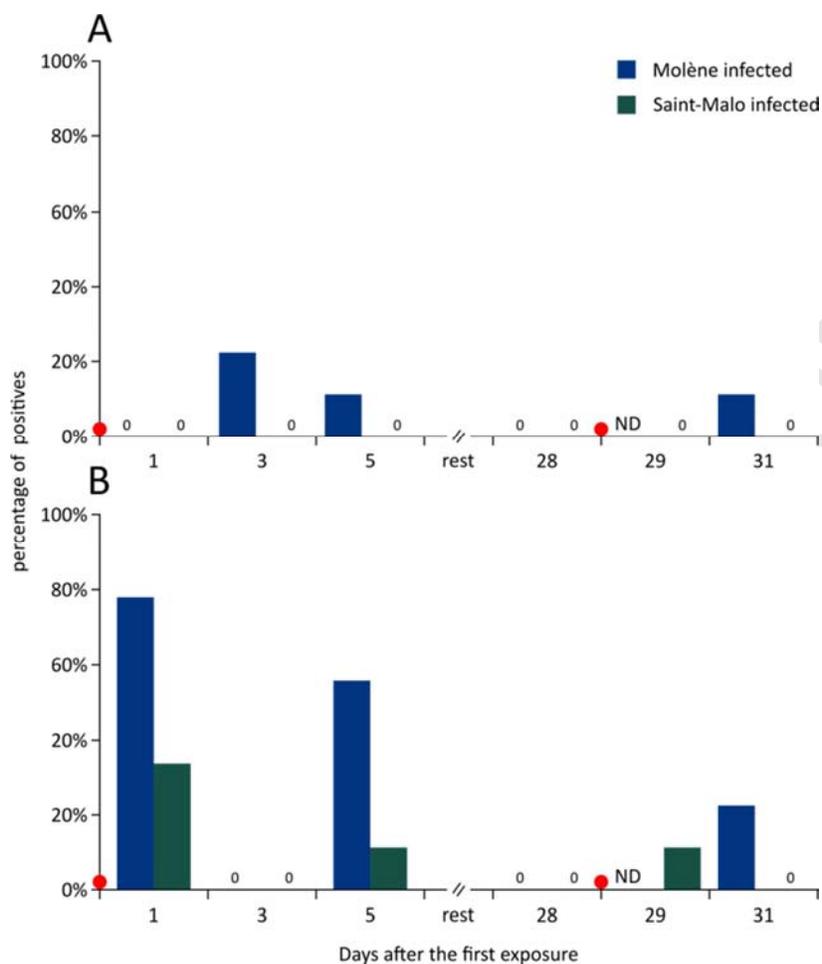
308 In the hemolymph, very few individuals were detected as positive for *V. harveyi*: 4 infected abalones  
 309 from Molène and only 1 from Saint-Malo across all time points combined. Two uninfected control individuals  
 310 (one in each population) were also detected as positive. *V. harveyi* was detected in the gills of a greater number  
 311 of individuals: 15 positives in Molène and 5 in Saint-Malo with *V. harveyi* being detected in 1 uninfected control  
 312 in the Molène population. The time points which exhibited the greatest number of positives were 1 day and 5  
 313 days after the first exposure. For all time points, the proportion of positive individuals was greater for Molène  
 314 than for Saint-Malo. A logistic regression was consequently performed only on the infected abalones by taking  
 315 into account 3 explanatory factors relating to the probability of abalones to be positive for *V. harveyi* on the  
 316 gills: the phagocytosis index, the THC and the populations. All three factors significantly influenced the odds of  
 317 abalone being positive for *V. harveyi*, with the odds of an abalone being positive for *V. harveyi* being 12.9 times  
 318 higher in abalones from Molène.  
 319

Table 1: Logistic regression examining the effect of the population(pop), the phagocytosis index (phago) and the total haemocytes count (THC), on the proportion of abalones positive to *V. harveyi* on the gills.  
 (\*) For THC, the estimate and odds ratio are calculated for an increase of 500 cells/ $\mu$ L.

320

Coefficients:

	Estimate	odds ratio	std. Error	z value	Pr(> z )	
(Intercept)	-5,02786	0,00655282	2.19997	-2.285	0.02229	*
pop	2,55707	12,8979708	0.80107	3.192	0.00141	**
phago	-0,17121	0,8426446	0.07558	-2.265	0.02350	*
THC (*)	1,30651	3,69326171	0.50927	2.565	0.01030	*



321

**Fig. 4:** Percentage of positive individuals for *V.harveyi* (n=9) in (A) haemolymph and (B) gills obtained by qPCR using specific primers and a TaqMan probe. The number 0 indicates that no individuals were found as positive at a given population and time point. Red dots indicate the timing of bacterial exposure. ND indicates that no data is available.

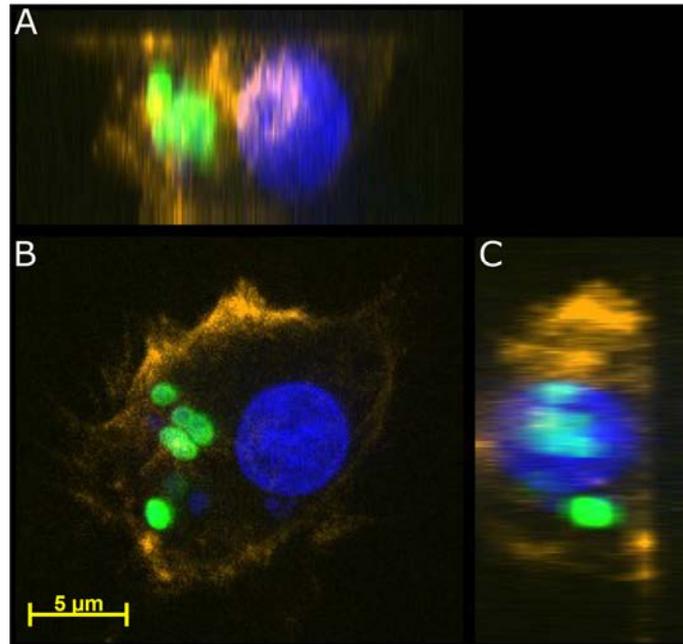
322

323 *3.5 Post-infection analyses:*324 *3.5.1 Fluorescence microscopy of phagocytosis*

325 In order to validate that *V. harveyi* is internalized rather than adhered externally to abalone  
 326 haemocytes, 3-dimensional fluorescence microscopy was carried out using individuals from Saint-Malo and  
 327 Molène. Three-dimensional fluorescence microscopy shows that *V. harveyi* is well phagocytosed by the  
 328 haemocytes of *H. tuberculata* from the two populations. In Fig 5, the nucleus of the haemocyte is shown in blue  
 329 and its cytoskeleton, delimiting the plasma membrane of haemocytes, is shown in orange. The green points  
 330 observed to the left of the nucleus and within the cell membranes correspond to GFP-labelled *V. harveyi*  
 331 located inside the cell. The flanking panels (Fig. 5 A, C), showing cross-sections of the haemocyte along the z-

332 and x-axes, confirm that bacteria are inside haemocyte cells and not merely at the surface. Similar images were  
 333 obtained using haemocytes from individuals from both Saint-Malo and Molène.

334



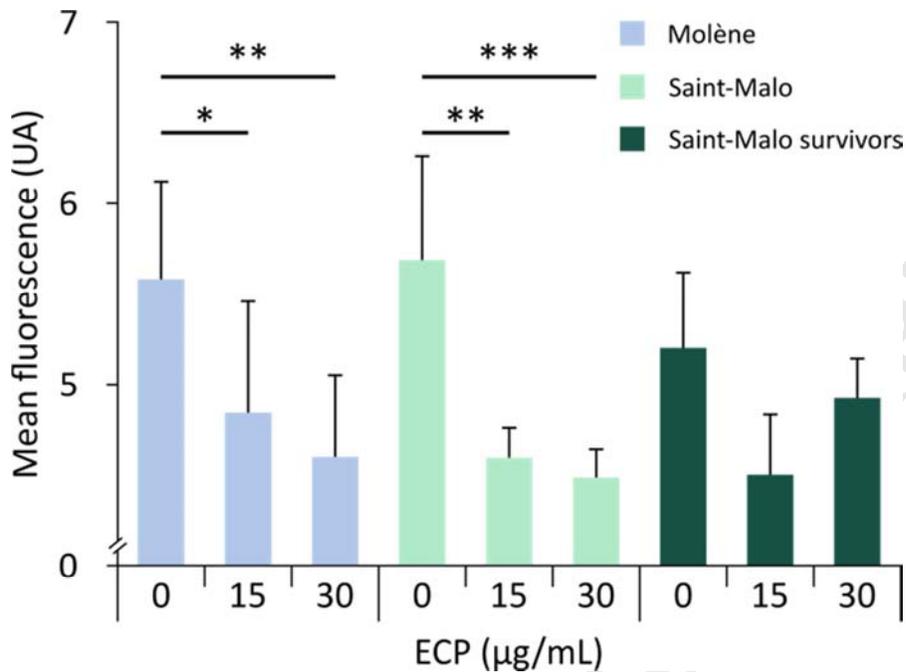
335

**Fig. 5:** 3-dimensional fluorescence microscopy (x60) pictures of a haemocyte (cytoskeleton in orange and nucleus in blue) which has phagocytosed GFP-labelled *V. harveyi* (green). The central picture (B) shows a reconstruction of 14 stacked fluorescence images. The flanking pictures show cross-sections compiled along the (A) z-axis and (C) x-axis.

336

### 337 3.5.2 Effect of the *V. harveyi* ECPs on the capacities of abalone to phagocyte this bacteria

338 Haemocytes of abalones from each population were exposed to 0, 15 and 30  $\mu\text{g}\cdot\text{ml}^{-1}$  of ECPs obtained from the  
 339 ORM4 strain of *V. harveyi*. Phagocytosis of GFP-labelled *V. harveyi* under exposure to ECPs was quantified two  
 340 months after the successive infections experiment (Fig. 6). A nested ANOVA showed no significant differences  
 341 between the Saint-Malo and Molène populations, but the factor ECPs exhibited a significant p-value < 0.001.  
 342 Thus, pairwise comparisons of ls-means were performed within each population in order to evaluate their  
 343 responses to ECPs treatments. A concentration of 30 $\mu\text{g}\cdot\text{ml}^{-1}$  ECPs showed a significant negative effect on the  
 344 phagocytosis index of uninfected individuals, with an inhibition of phagocytosis of 19% for abalone from  
 345 Molène and 22.8% for Saint-Malo. Abalones from Saint-Malo having survived the successive infections showed  
 346 no significant difference in phagocytosis when exposed to 0, 15 and 30  $\mu\text{g}\cdot\text{ml}^{-1}$  of ECPs.



347

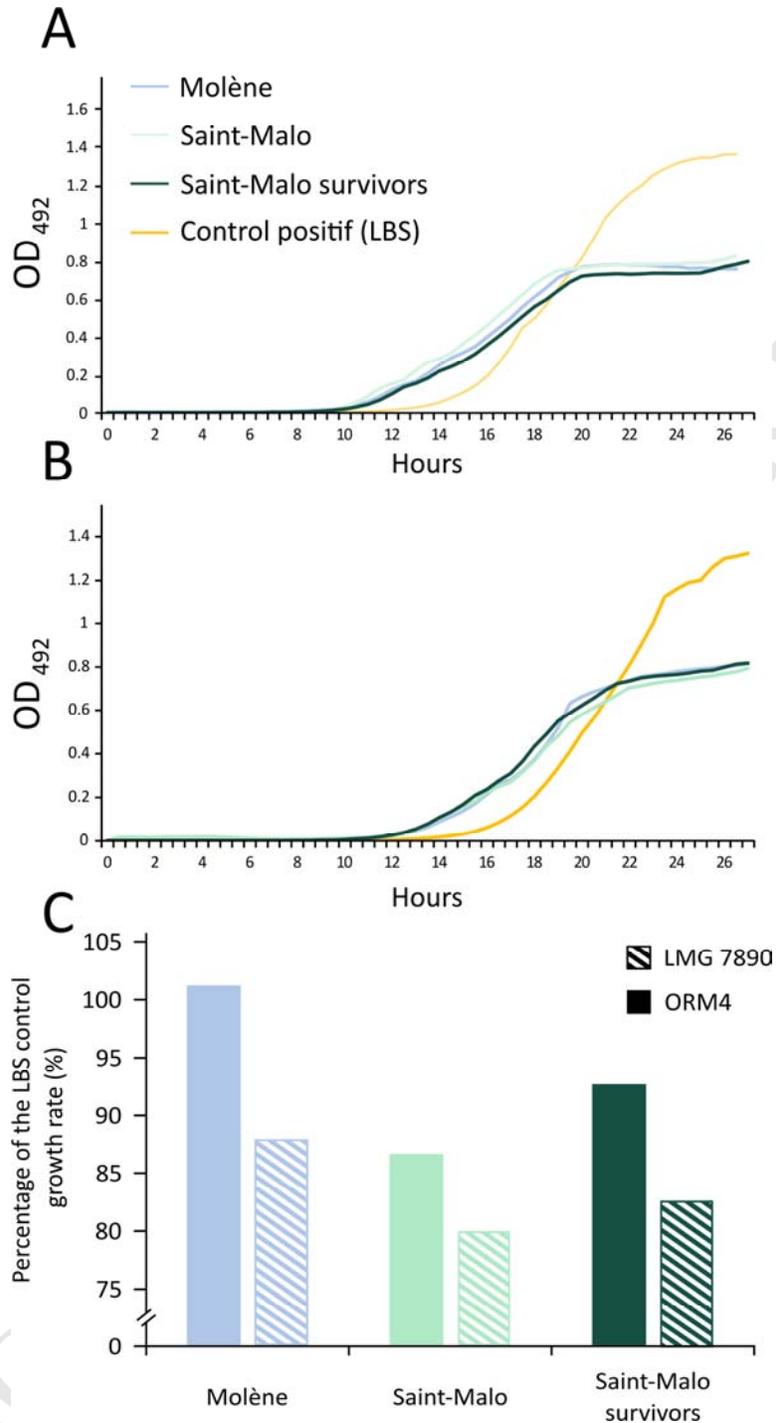
**Fig. 6:** Impact of two concentrations of extracellular products of *V. harveyi* (15µg/mL and 30 µg/mL) on phagocytosis of GFP-labelled bacteria. Values are the means of green fluorescence emitted by haemocytes. \* indicates values that are significantly different from condition without ECPs for a pairwise comparison of the least-squares means (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

348

### 349 3.5.3 Ability of *V. harveyi* to grow in acellular fraction of abalones hemolymph

350 The growth of two strains of *V. harveyi* (LMG7890 and ORM4) in the acellular fraction of the  
 351 hemolymph from abalone of Saint-Malo and Molène were followed during 42 hours (Fig. 7). In order to have  
 352 the necessary volume of serum, hemolymph of five individuals was pooled for each population. The two  
 353 bacterial strains tested began their growth at approximately 2-3 hours earlier than in LBS, the positive control.  
 354 Growth rate was faster in the pathogenic strain (ORM4) relative to the non-pathogenic (LMG7890). Moreover,  
 355 the ability of *V. harveyi* to grow in abalone serum was lower in Saint-Malo (86% of the maximum growth rate  
 356 observed in LBS for uninfected abalone and 92% for survivors), while the growth rate in the serum of abalone  
 357 from Molène was nearly the same as in the LBS positive control (101% of the rate observed in LBS).

358



**Fig. 7:** Growth curves of the (A) non-virulent strain LMG7890 and (B) the virulent strain ORM4 in the serum of abalones. The growth of the bacteria in LBS was used as a positive control. (C) Growth rate of the LMG7890 and ORM4 strains in abalone serum are expressed as a percentage of the maximum growth rate in the LBS control.

359

360

361

#### 4. Discussion

362 Populations exposed to contrasting environmental conditions and having different disease occurrences  
363 can evolve different susceptibilities against a particular pathogen. Based on this hypothesis, two populations of  
364 *H. tuberculata* were chosen to examine how response to infection to *V. harveyi* could vary in abalone of  
365 different origins. In Saint-Malo, where the average sea water temperatures exceed 17°C during the summer  
366 spawning period, conditions are favorable for disease development and indeed, this population has been  
367 frequently impacted by disease [10]. On the other hand, mortality has never been reported in Molène and the  
368 surrounding region, where temperatures of 17°C are rarely observed[9]. Successive infections conducted with  
369 abalones from each of these two natural populations showed marked differences in survival. Following 24 days  
370 after a first exposure to *V. harveyi*, a survival rate of 51% was observed for abalone from Molène and 95% for  
371 abalone from Saint-Malo (Fig. 1). Interestingly, the survival rate for Saint-Malo was not statistically different  
372 from that of uninfected controls. Our experimental infections confirm the hypothesis that abalone from a site  
373 that has experienced recurrent mortality (Saint-Malo) shows improved survival following infection with *V.*  
374 *harveyi*. According to Coustau and Théron (2004), resistance is defined as a relative term which indicates that a  
375 group exhibits a significantly better ability to prevent infection by a specific pathogen. Thus, all subsequent  
376 analyses were performed to identify mechanisms which could explain the resistance to the disease observed in  
377 abalone from Saint-Malo.

378 Differences in survival of *H. tuberculata* following successive infection with *V. harveyi* ( $10^6$  bacteria/mL;  
379 19°C) has been previously observed in farmed abalones [17]. Survival rate improved from 36% after a first  
380 infection to 56% following a second exposure, revealing different levels of resistance to the disease within the  
381 farmed population and a better ability to resist a second infection. While reduced mortality in this experiment  
382 may have been due to immune priming, the enhanced resistance observed at the second exposure could also  
383 be explained as an elimination of susceptible phenotypes following the first infection. In order to isolate a  
384 priming effect, improved response to the disease needs to be observed in conditions where potentially  
385 susceptible phenotypes are not eliminated at a first infection. Therefore, in the present experiment, mild  
386 infection conditions in term of both temperature (18°C) and bacterial concentration ( $10^4$  bacteria/mL) were  
387 used to avoid mortality after a first exposure to *V. harveyi*, which was the case for the Saint-Malo population.  
388 Despite these infection conditions, survival was low in the susceptible population of Molène. Thus, these  
389 infection conditions allowed on one hand, to discriminate the two populations in term of resistance, and on the

390 other hand, to discern which parameters allowed improved resistance to the disease in abalone from Saint-  
391 Malo.

392 After the first infection with *V. harveyi*, individuals from both Saint-Malo and Molène suffered an  
393 important drop in phagocytosis (~40% compared to the control) after the first day of exposure, followed by a  
394 recovery of this activity by the third day, showing a similar response between the two populations in cellular  
395 immunity during the first infection (Fig. 3). The reduction in phagocytosis index could be explained by the  
396 saturation of a high proportion of haemocytes following active phagocytosis of *V. harveyi* in the early stages of  
397 exposure, resulting in less efficient bead engulfment by 24 hours post-infection. Alternatively, the observed  
398 reduction in phagocytosis could be interpreted as an inhibition of phagocytosis. Previous studies have shown  
399 that the ORM4 strain can perturb the MAPK signaling pathway by inhibiting phosphorylation of the p38 MAPK,  
400 leading to inhibition of phagocytosis compared to the non-pathogenic strain of *V. harveyi* (LM7890)[16].  
401 Moreover, a significant decrease in phagocytosis index 24 hours after exposure to *V. harveyi* is linked with a  
402 downregulation of clathrin, a protein involved in endocytosis [15]. Therefore, the lower phagocytosis index  
403 observed is likely due to an inhibition of phagocytosis induced by the pathogen.

404 Interestingly, no significant decrease in phagocytosis was observed 1 day after the second exposure for  
405 abalones from Saint-Malo (Fig. 3A). The response of this population to the second infection can be interpreted  
406 as an immune priming effect. The survival rates observed in the control and infected conditions in Saint-Malo  
407 were similar, supporting the interpretation that the improved response to an infection can be due to a priming  
408 effect rather than an elimination of susceptible phenotypes. For the Molène population, low survival rates  
409 following the first infection preclude such interpretation.

410 Immune priming allows invertebrates to show improved survival to a pathogen following a first  
411 infection. This mechanism is now known in several insect species [30–32] and the freshwater snail  
412 *Biomphalaria glabrata* [20,33]. In the marine realm, immune priming was first examined in copepods [34].  
413 More recently, examples among a few marine molluscs have also been documented: *Chlamys farreri* [35],  
414 *Mytilus galloprovincialis* [36] and *Crassostrea gigas* [37]. In the gastropod *Biomphalaria glabrata*, a species  
415 phylogenetically close to *H. tuberculata*, a first exposure to the trematode *Schistosoma mansoni* conferred an  
416 immune priming effect which led to complete protection, such that a secondary infection exhibited animals  
417 with a parasite prevalence of 0% for primed individuals compared to 100% for unprimed [20]. In the Pacific  
418 oyster *C. gigas*, a more acute and rapid immune response in term of phagocytosis and hematopoiesis was

419 observed after being primed with heat-killed *Vibrio splendidus* 7 days before the infection [35]. Since  
420 phagocytosis is usually the first response of the host against the pathogen, inhibition of this mechanism is a  
421 widespread strategy among pathogens to persist inside the host tissues [38]. Early phagocytosis response can  
422 then be crucial for the resistance of animals against infection and septicemia. In the case of *H. tuberculata*, the  
423 first exposure can act as an immune treatment that prevents future phagocytosis inhibition in abalone, thereby  
424 improving the early response to a subsequent exposure. A priming effect could allow the abalone immune  
425 system to be stimulated in the field at the beginning of the mortality season, thus enhancing protection for the  
426 rest of the critical period.

427         Because of the importance of the phagocytic response, and its implication in priming effect, this  
428 immune mechanism has been further examined. During the experimental infections, host phagocytosis was  
429 quantified using fluorescent beads. This commonly used approach [16] shows the activity of haemocytes rather  
430 than the actual ability to phagocytose a specific bacterium cell. While actual internalization of *V. harveyi* cells  
431 has previously been shown in primary cultured cells of farmed abalone haemolymph and gills [26], 3D  
432 fluorescent microscopy was used to confirm that this was the case in the freshly collected haemolymph from  
433 abalone from both Saint-Malo and Molène. Fluorescent microscopy shows that GFP-labelled bacterial cells are  
434 clearly observed inside the haemocytes, providing unquestionable evidence of internalization of *V. harveyi* by  
435 the haemocytes of *H. tuberculata* (Fig. 5) and confirming that subsequent flow cytometry measurements made  
436 with GFP-labelled *V. harveyi* quantifies actual phagocytosis. Phagocytosis of GFP-labelled bacteria performed  
437 two months after the infection experiment on uninfected individuals exhibited similar responses between the  
438 two populations. Abalones from Saint-Malo and Molène showed the same phagocytosis capacity and the same  
439 response to the ECPs. Indeed, the two populations suffer a phagocytosis inhibition of about 20% when exposed  
440 to  $30 \mu\text{g}\cdot\text{ml}^{-1}$  of ECPs relative to the  $0 \mu\text{g}\cdot\text{ml}^{-1}$  controls (Fig. 6). However, for the abalone from Saint-Malo  
441 surviving the successive infections, no statistical difference was observed between phagocytosis index exposed  
442 to concentrations of  $0 \mu\text{g}$ ,  $15 \mu\text{g}\cdot\text{ml}^{-1}$  and  $30 \mu\text{g}\cdot\text{ml}^{-1}$  of ECPs, suggesting a potential long-term priming effect  
443 against the inhibition of phagocytosis induced by ECPs. This result indicates that the protection against  
444 phagocytosis inhibition induced by the first exposure has persisted for over two months.

445         Our results are the first to indicate the existence of immune priming in abalones, however, the present  
446 study does not differentiate between the two possible types of priming. Immune priming in invertebrates  
447 occurs either as a sustained response of immune mechanisms which prevents a subsequent attack, or via a

448 specific response which allows recognition of the pathogen thus inducing a more intensive and rapid immune  
449 response [20]. Future work addressing whether sustained response or specificity of response is present in  
450 abalone could further our understanding of how immune priming acts in this species. For example, the injection  
451 of heat-killed bacteria could address whether immune priming is a specific response in *H. tuberculata*, as  
452 performed with the Pacific oyster [37]. Enhanced phagocytosis was observed only in oysters injected with heat-  
453 killed *V. splendidus*, but not with 4 other species of bacteria, suggesting specific recognition of this pathogen.  
454 The injection of heat-killed bacteria would also allow the induction of a more intensive immune effect by  
455 delivering higher doses of the pathogen. The infection performed in the present study was weak to avoid  
456 mortality, possibly leading to a partial or diminished immune priming response.

457 Sustained immune response is another possible mechanism of immune priming. The pathogenic strain  
458 of *V. harveyi* ORM4 is able to avoid the bactericidal response of abalone through an inhibition of the activity of  
459 the p38 MAPK, a MAP kinase which is thought to trigger a number of immune responses such as phagocytosis  
460 or the secretion of reactive oxygen species [16]. This kind of virulence has also been shown in other marine  
461 models. For example, the secretion of a metalloprotease by *Vibrio aestuarianus*, a pathogen of the Pacific  
462 oyster *C. gigas*, inhibits, among other immune parameters, phagocytosis [39]. Proteases secreted by the  
463 pathogen are a common mechanisms for the inhibition of phagocytosis, but can be counter-acted by protease  
464 inhibitors produced by the host [40]. In the disk abalone *Haliotis discus discus*, three types of clade B serine  
465 protease inhibitors are expressed in haemocytes following injection of *V. parahaemolyticus* or of LPS [41].  
466 Sustained synthesis of protease inhibitors by *H. tuberculata* may therefore be a possible explanation of the  
467 long-term protection against phagocytosis inhibition. Future work quantifying protease inhibitors following  
468 successive infections with *V. harveyi* could confirm this hypothesis.

469 Humoral effectors may also contribute to the resistance of the Saint-Malo abalone to *V. harveyi*  
470 infection. Marine invertebrates possess a large set of antimicrobial peptides that can counteract bacterial  
471 growth [42]. Hemocyanin can also have strong antimicrobial activity [43], while other factors can limit bacterial  
472 growth by sequestering or limiting the availability of nutrients such as iron [44]. In the European abalone, the  
473 onset of growth of the two bacterial strains occurred 2-3 hours earlier in the serum of abalone than the LBS  
474 control, irrespective of the population, showing potential activators of bacterial growth may be present in the  
475 abalone serum. Moreover, bacterial growth rate was greater for the virulent strain of *V. harveyi* (ORM4)  
476 compared to the non-virulent strain LMG 7890 (Fig. 7). However, maximum growth rate of the virulent strain

477 ORM4 was lower in the haemolymph of Saint-Malo compared to Molène, indicating that the serum of abalone  
478 from Saint-Malo is less favorable for ORM4 growth. Therefore, resistance to *V. harveyi* in abalone from Saint-  
479 Malo may in part be explained by the ability to slow down bacterial growth within the serum. Host fluids can  
480 have significant effects in growth and gene expression of bacteria [45,46]. For example, the pedal mucus of the  
481 small abalone *Haliotis diversicolor* has been showed to induce the formation of a biofilm by *Vibrio alginolyticus*  
482 and to enhance the density of bacteria [46]. In the present work, bacterial growth in the serum was measured  
483 two months after the successive infections; it is possible that different responses may be observed during  
484 infection with *V. harveyi*.

485 The most striking differences between the resistant and susceptible populations were observed in the  
486 detection of *V. harveyi* in the haemolymph and the gills of abalone. In the haemolymph, *V. harveyi* was  
487 detected in only 5 individuals, all from the Molène population (Fig. 4A). Despite the low survival rate in abalone  
488 from Molène, the small number of individuals positive for *V. harveyi* can be explained by the rapid growth rate  
489 of *V. harveyi* in abalone serum (~10 hours, see Fig 7), rendering the time frame to detect the bacteria in  
490 hemolymph (between the beginning of exponential phase and septicemia) very short. It is nevertheless  
491 interesting to note that all individuals for which *V. harveyi* was detected were from Molène, suggesting that *V.*  
492 *harveyi* is better able to penetrate the haemolymph of abalone from this population. The results of THC  
493 support this interpretation. Although no differences in THC were observed between infected abalones and  
494 uninfected controls in the Saint-Malo population, abalone from Molène showed a significant increase of the  
495 number of circulating haemocytes after 24 hours of exposure to *V. harveyi* (Fig. 2). This likely denotes an  
496 inflammatory response by a recruitment of haemocytes in the hemocoel suggesting greater presence of *V.*  
497 *harveyi* in this compartment in abalone from Molène.

498 Detection of *V. harveyi* in the gills was significantly greater in abalone from Molène compared to Saint-  
499 Malo (Fig. 4B), and a binomial regression showed that the detection of *V. harveyi* on the gills was correlated  
500 with an increase of THC and a decrease of phagocytosis index (Table. 1). These findings indicate that even if the  
501 bacterium is not detected in the haemolymph, its presence in the gills already induces an immune response.  
502 The portal of entry of *V. harveyi* is the gills of abalones [47], where previous work has shown that bacterial  
503 density can be 5-fold greater in the gills compared to other tissues 6 hours after exposure. The small number of  
504 individuals which were positive for *V. harveyi* in the gills among abalone from Saint-Malo suggests that an  
505 important part of the resistance of this population may depend on the ability to prevent the settlement and

506 penetration of bacteria in the gills. The ability to adhere to the portal of entry of the host can be essential for  
507 the virulence of a bacterium. This is the case of *Flavobacterium columnare* and *Yersinia ruckeri*, for which all  
508 known virulent strains are able to adhere to the gills of their respective hosts, whereas non-virulent strains  
509 cannot [48,49]. Preventing settlement of bacteria on the gills may be an important defense mechanism against  
510 disease. Other strategies can also be used to counteract the settlement of bacteria on the gills of marine  
511 invertebrates, such as the localized production of lysozyme or antimicrobial peptides. In the penaid shrimp  
512 *Marsupenaeus japonicas*, lysozyme expression and antimicrobial activity are elevated in the gills [50].  
513 Moreover, an antimicrobial peptide expressed only in gills has been discovered in the abalone *Haliotis discus*  
514 [51]. Since the gills may be important in the resistance to *V. harveyi* infection, future work comparing potential  
515 antimicrobial or anti-adherent activity in the gills of abalone from the two populations may help to identify the  
516 mechanisms by which abalone from Saint-Malo have enhanced resistance against *V. harveyi*.

517 Surprisingly, no individuals were positive for *V. harveyi* in the gills 3 days after the first exposure in  
518 both populations. This is possibly due to the fact that bacterial concentrations fluctuate over the course of the  
519 experimental infection, as was quantified in similar experiments [22]. Thus, bacterial concentrations at this  
520 given time point may have fallen below the detection limit.

521

## 522 5. Conclusions

523 This study shows the differential resistance between the two populations of *H. tuberculata* against *V.*  
524 *harveyi* and the comparisons between these two populations identified a number of resistance effectors.  
525 Abalone haemolymph exhibited weak defenses against the bacteria, and are presumably insufficient to contain  
526 a septicemia, although phagocytosis and limitation of bacterium growth in the serum are two possible  
527 resistance mechanisms. On the other hand, the significant differences observed in detection of *V. harveyi* in the  
528 gills point towards an important implication of the gills in the resistance of the Saint-Malo population. Our  
529 results show the first evidence of immune priming in *Haliotis tuberculata* and the enhanced capacity of  
530 phagocytosis at the second infection demonstrate a potential importance of cellular response against *V.*  
531 *harveyi*. A synergistic interaction among effectors in the gills and haemolymph likely lead to disease resistance.  
532 Further work is needed to understand precisely how the population of Saint-Malo resists infection and to find  
533 the gills effectors that counteract the settlement of *V. harveyi* in abalone gills.

534

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542

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679 **Captions**

680 **Figure 1:** Kaplan-Meier survival rate following the first exposure of the two populations to  $10^4$   
681 bacteria/mL during 24 hours at  $18^\circ\text{C}$

682 **Figure 2:** Total haemocytes count (THC) during the two exposures of (A) Saint-Malo and (B) Molène.  
683 Red dots indicate the exposures. ND indicates that no data is available. \* indicates values that are  
684 significantly different from the control for a pairwise comparison of the least-squares means ( $p <$   
685  $0.05$ ).

686 **Figure 3:** Phagocytosis index based on micro-beads engulfment (percentage of haemocytes  
687 containing three or more fluorescent beads relative to total haemocytes) during two successive  
688 infections of abalone from (A) Saint-Malo and (B) Molène. Red dots indicate the timing of bacterial  
689 exposure. ND indicates that no data is available. \* indicates values that are significantly different from  
690 the control for a pairwise comparison of the least-squares means ( $p < 0.05$ ).

691 **Figure 4:** Percentage of positive individuals for *V.harveyi* ( $n=9$ ) in (A) haemolymph and (B) gills  
692 obtained by qPCR using specific primers and a TaqMan probe. The number 0 indicates that no  
693 individuals were found as positive at a given population and time point. Red dots indicate the timing  
694 of bacterial exposure. ND indicates that no data is available.

695 **Figure 5:** 3-dimensional fluorescence microscopy (x60) pictures of a haemocyte (cytoskeleton in  
696 orange and nucleus in blue) which has phagocytosed GFP-labelled *V. harveyi* (green). The central  
697 picture (B) shows a reconstruction of 14 stacked fluorescence images. The flanking pictures show  
698 cross-sections compiled along the (A) z-axis and (C) x-axis.

699 **Figure 6:** Impact of two concentrations of extracellular products of *V. harveyi* ( $15\mu\text{g/mL}$  and  $30$   
700  $\mu\text{g/mL}$ ) on phagocytosis of GFP-labelled bacteria. Values are the means of green fluorescence  
701 emitted by haemocytes. \* indicates values that are significantly different from condition without  
702 ECPs for a pairwise comparison of the least-squares means (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

703 **Figure 7:** Growth curves of the (A) non-virulent strain LMG7890 and (B) the virulent strain ORM4 in

704 the serum of abalones. The growth of the bacteria in LBS was used as a positive control. (C) Growth  
705 rate of the LMH7890 and ORM4 strains in abalone serum are expressed as a percentage of the  
706 maximum growth rate in the LBS control.  
707

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