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## Immune responses of phenoloxidase and superoxide dismutase in the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis* – Part I: Spatio-temporal evolution of enzymes' activities post-infection

Le Bris Cedric<sup>1,\*</sup>, Richard Gaelle<sup>1</sup>, Paillard Christine<sup>1</sup>, Lambert Christophe<sup>1</sup>, Segueineau Catherine<sup>1</sup>, Gauthier Olivier<sup>1</sup>, Pernet Fabrice<sup>2</sup>, Guerard Fabienne<sup>1</sup>

<sup>1</sup> UMR 6539 CNRS UBO IRD IFREMER, LEMAR, IUEM, UBO, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest-Iroise, Rue Dumont d'Urville, 29280 Plouzané, France

<sup>2</sup> UMR 6539 CNRS UBO IRD IFREMER, LEMAR, IUEM, UBO, Ifremer, Laboratoire de Physiologie des Invertébrés, Technopôle Brest-Iroise BP 70 29280, Plouzané, France

\* Corresponding author : Cédric Le Bris, email address : [cedric.lebris@yahoo.fr](mailto:cedric.lebris@yahoo.fr)

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### Abstract :

Manila clams, *Venerupis philippinarum* (Adams and Reeve, 1850), were experimentally challenged with two *Vibrio tapetis* strains: CECT4600T, the causative agent of Brown Ring Disease (BRD); and LP2 supposedly non-pathogenic in *V. philippinarum*. Changes in phenoloxidase (PO) and superoxide dismutase (SOD), two major enzymes involved in immunity, were studied in two tissues, the mantle and hemolymph for 30 days after infection in the extrapallial cavity. Bacterial infection in *V. philippinarum* resulted in modulation of PO and SOD activities that was both tissue- and time-dependent. A response at early times was detected in the mantle and was associated with significant increases in PO and SOD activities in LP2- and CECT4600T-challenged clams 36h post injection. This first response in the mantle could be explained by the proximity to the injection region (extrapallial cavity). In the hemolymph the response occurred at later times and was associated with an increase in PO activity and a decrease in SOD activity. As hemolymph is a circulating fluid, this response delay could be due to an "integration time" needed by the organism to counteract the infection. Injections also impacted PO and SOD activities in both tissues and confirmed a difference in pathogenicity between the two *V. tapetis* strains.

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## Highlights

► *Venerupis philippinarum* infection with two *Vibrio tapetis* strains (CECT4600 and LP2). ► Modulation of PO and SOD activities after infections was tissue- and time-dependent. ► PO and SOD activities increased 36 h post injection in the mantle. ► PO activity increased and SOD activity decreased in later times in hemolymph. ► PO and SOD activity levels confirmed the strain pathogenicity difference.

**Keywords** : *Venerupis philippinarum*, *Vibrio tapetis*, Brown Ring Disease, Immunity, Phenoloxidase, Superoxide Dismutase

## 1. Introduction

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The Manila clam, *Venerupis philippinarum* (Adams and Reeve, 1850) was introduced to France from the USA in 1972 for aquaculture purposes, because its growth is faster than that

34 of the European clam *Venerupis decussata* (Linnaeus, 1758) [1]. Since 1987, the species has  
35 been affected by brown ring disease (BRD) caused by the pathogenic gram-negative  
36 bacterium *Vibrio tapetis* [2]. BRD is diagnosed by the formation of a brown deposit of  
37 conchiolin at the edge of the mantle on the inner shell of the clam [3]. During the  
38 development of the disease, the bacterium first colonizes the periostracal lamina, and then in  
39 some conditions (lesions, starvation) tissues and finally moves into the circulatory system,  
40 leading to a systemic infection and the organism's death; the effects of BRD on Manila clams  
41 have been reviewed by Paillard [4]. BRD has decimated clam populations, first in Brittany  
42 (France) and spreading along French and European coasts [3]. Since the isolation of the  
43 pathogenic bacterium (*V. tapetis* CECT4600<sup>T</sup>) in the Manila clam from Landeda (Brittany,  
44 France) in 1990 [5, 6], around 40 different *V. tapetis* strains have been isolated in various  
45 mollusks and fishes in France [5], England [7, 8], Spain [9], Norway [10], Scotland [8] and  
46 Japan [11]. Pathogenicity of the majority of these strains has been evaluated, and *V. tapetis*  
47 CECT4600<sup>T</sup> was shown to be the most pathogenic strain to *V. philippinarum* [12, 13]. To  
48 prevent mortalities caused by BRD it is necessary to understand both the mechanisms by  
49 which the pathogenic bacteria act and equally how the host immune system functions. Many  
50 studies have been conducted on clam immune responses during bacterial infection, and it has  
51 been shown that the occurrence of *V. tapetis* in the hemolymph leads to a decrease in size and  
52 complexity of granulocytes [14] and to a loss of hemocyte adhesion capacities because, these  
53 cells become rounded and lose their pseudopodia [12]. More recently it was demonstrated that  
54 *V. tapetis* occurrence in hemolymph significantly increases nitric oxide production in a dose-  
55 dependent manner [15]. Most of the studies on *V. philippinarum* immune response to *V.*  
56 *tapetis* infection have focused on cellular immunity, in particular hemocytes [7, 16-19].  
57 However, invertebrate immunity can be divided into cellular and humoral immune response  
58 [20]. Hence, host immunity, and its potential defense capability against pathogen agents,  
59 could also be investigated through the nature and activity of endogenous enzymes [21].  
60 Phenoloxidase (PO), which is one of these humoral immune enzymes, is often monitored  
61 because of its key role in invertebrate immune systems and especially in the activation of the  
62 melanization cascade [22]. In fact POs (E.C. 1.14.18.1, E.C. 1.10.3.1 and E.C. 1.10.3.2)  
63 oxidize phenolic compounds into corresponding quinones and then into melanin and its  
64 derivatives [23, 24] which have fungistatic, bacteriostatic and antiviral properties [25]. For  
65 example, in the oyster *Saccostrea glomerata*, a negative correlation was found between PO

66 activity level and the level of infection by *Marteilia sydneyi* which is responsible for QX  
67 disease [26]. Indeed, a QX resistance breeding program developed along the Australian East  
68 coast has selected oysters with enhanced PO activity [27]. PO activity in *V. philippinarum*  
69 hemolymph has been biochemically characterized [28, 29] but variations in PO after infection  
70 has, to date, never been followed. Superoxide dismutase (SOD) (E.C. 1.15.1.1), like PO, is an  
71 oxidoreductase that contributes towards innate immunity by preventing the accumulation of  
72 Reactive Oxygen Species (ROS) in the organism, particularly superoxide anions ( $O_2^-$ ). In  
73 marine bivalves, ROS are generated in larger proportions as the organism is exposed to biotic  
74 challenges such as *Vibrio* extracellular products [30, 31]. ROS are known to exhibit  
75 antimicrobial properties but overconcentration can lead to cellular damages: the antioxidant  
76 paradox [32]. In order to control the cell redox status, antioxidant enzymes are recruited to  
77 diminish ROS concentration. Intracellular SOD gene expression analyses performed in  
78 Manila clams infected with *Vibrio* species (*V. anguillarum*, *V. tapetis*) showed time-  
79 dependent variations over short periods (96 hours maximum) [33, 34], suggesting the  
80 regulation of this protein and its involvement in innate immunity of *V. philippinarum*.  
81 Nevertheless, SOD has not been studied biochemically in *Vibrio*-challenged clams so far, and  
82 it is interesting to focus on biological activities of PO and SOD over longer periods (up to 30  
83 days after injection) since BRD symptoms require time to develop after infection.  
84 Thus, the aim of this study was to investigate the effects *V. tapetis* infection on immune  
85 enzymatic activities in *V. philippinarum*. Clams were infected with two different *V. tapetis*  
86 strains and monitored for 30 days after infection. More precisely, our study compared PO and  
87 SOD immune activity, in different compartments, of clams injected with two different *V.*  
88 *tapetis* strains, considered more or less virulent, or with Sterile Sea Water (SSW) as a control.

## 89 **2-Materials and methods**

### 90 **2-1-Biological material and acclimation procedure**

91 Around 600 adult *V. philippinarum*, 39.80 mm  $\pm$  2.60 (SD) long were collected from Ile  
92 Tudy, Brittany (France), by SATMAR (Aquaculture Company) in October 2012 and  
93 transferred to the laboratory. These clams were divided between 9 aerated 75L tanks at 15.3  $\pm$   
94 0.64°C and acclimated for one week before injections. The 9 tanks correspond to the three  
95 conditions (Two *V. tapetis* strains and one control) made in triplicate.

## 96 2-2-Bacterial strains

97 For the bacterial challenge experiments, two *V. tapetis* strains were used: *V. tapetis*  
98 CECT4600<sup>T</sup> (also known as CIP 104856) which was isolated from diseased *V. philippinarum*  
99 from Brittany, France in 1990 [6, 35]; and LP2, isolated from the fish *Symphodus melops* in  
100 Norway in 1999 [10], and previously considered as non-pathogenic to *V. philippinarum* after  
101 *in vivo* pallial cavity inoculation or *in vitro* biotests [12, 13]. These strains were grown in  
102 Zobell's medium overnight at 18°C and bacterial solution concentrations were determined by  
103 spectrophotometry at 490 nm ( $[C] = \text{O.D.} \times 1.3 \times 10^9 - 3.6 \times 10^7$ ).

## 104 2-3-Experimental injections

105  
106 Before experimental injections, 30 clams were sacrificed in order to check their asymptomatic  
107 status. Twelve hours before injections, animals were removed from their tank and only  
108 replaced in 15°C sea water just before the injections to facilitate their opening. Experimental  
109 infections were carried out by injecting 100 µL of *V. tapetis* suspension ( $10^6 \text{ CFU.mL}^{-1}$ ) per  
110 individual into the extrapallial cavity with sterile needles (25G 0.5 × 16 mm) fitted onto 1mL  
111 sterile syringes. The needle was introduced in the extrapallial cavity under the mantle. Control  
112 clams were inoculated, in the same manner, with 100µL of sterile sea water (SSW) in the  
113 extrapallial cavity. After the injections, animals were kept for 6 hours out of sea water before  
114 being put back into their aerated tanks to keep the bacteria and favor their colonization to the  
115 periostracal lamina.

## 116 2-4-Fluid and tissue collection

117 **Individuals were sampled before the injections (T0), and at 36 hours, 72 hours, 7 days, 14**  
118 **days and 30 days post-injection (p.i.). On each sampling occasion 5 individuals were**  
119 **sampled from each individual tank, yielding a total of 15 individuals per condition at**  
120 **every sampling time.**

### 121 2-4-1- Hemolymph and extrapallial fluid collection

122 Hemolymph of each sampled clam was withdrawn from the adductor muscle as described by  
123 Auffret & Oubella [36]. Collected hemolymph was centrifuged at 785g for 10 minutes at 4°C,  
124 to separate the hemocytes from the serum. The resulting serum was stored at -80°C until  
125 enzymatic and protein assays. After hemolymph collection, the Extrapallial Fluid (EF) was  
126 collected from each valve by opening the valves and inserting a second sterile needle (25G

127 0.5 × 16 mm), fitted on a sterile syringe, between the mantle and shell in the sinusal and  
128 central compartments. The EF was then centrifuged at 785g for 10 minutes at 4°C to separate  
129 the cellular and acellular fractions (serum). This serum was stored at -80°C until enzymatic  
130 and protein assays.

#### 131 **2-4-2-Tissue collection and protein extraction**

132 Both right and left mantle were dissected and immediately frozen in liquid nitrogen. Samples  
133 were stored at -80°C until being powderized in a Retsch MM 400 blender by grinding with  
134 liquid nitrogen. The powder obtained was stored at -80°C. Before use, samples were  
135 resuspended in 500µL of lysis buffer [37]. The resuspended powder was then homogenized  
136 using Ultra-Turrax (Modele PRO 200) and centrifuged at 10 000g for 45 minutes at 4°C.  
137 Enzymatic and protein assays were carried out on the resulting supernatant.

#### 138 **2-5-Protein determination**

139 The total protein content of *V. philippinarum* serum, extrapallial fluid and mantle lysate  
140 supernatant was determined by the Bradford method [38] using BioRad Protein Assay Dye  
141 Reagent Concentrate (BioRad France) and bovine serum albumin (BSA) as the protein  
142 standard.

#### 143 **2-6-Enzyme activity assays**

##### 144 **2-6-1-Phenoloxidase assays**

145 PO activity was assayed spectrophotometrically in 96-well microplates (Greiner 96-F-  
146 bottom), by recording the formation of quinones according to the protocol of Le Bris *et al.*  
147 [29]. Briefly, 50µL of Tris-HCl buffer (0.10M, pH 8.0) were added to 50µL of enzymatic  
148 solution (Hemolymph and EF serum and grinded mantle supernatant). This mixture was  
149 incubated for 10 minutes at 25°C, before 100µL 0.04M L-3,4-dihydroxyphenylalanine (L-  
150 DOPA) were added to each well. L-DOPA is common substrate for the three PO subclasses.  
151 PO activity was then monitored for 30 minutes following the increase of absorbance at 492nm  
152 (using POLARstar Omega – BMG Labtech). At the same time, the spontaneous oxidation of  
153 L-DOPA was measured and the values obtained were subtracted from the test values. The PO  
154 specific activities in international unit (U.I.) per milligram of total protein were calculated as  
155 follows:

156 PO specific activity (U.I.mg protein<sup>-1</sup>) = ( $\Delta A \cdot \text{min}^{-1} \times \text{dilution factor}$ ) / Total protein  
157 concentration

158 Where  $\Delta A \cdot \text{min}^{-1}$  is the value of the increment of absorbance per minute, dilution factor is the  
159 sample's factor of dilution, and total protein concentration is the sample's protein  
160 concentration.

### 161 *2-6-2-Superoxide dismutase assays*

162 Total superoxide dismutase (SOD: EC 1.15.1.1) activity was assayed spectrophotometrically  
163 in 96-well microplates (Greiner 96-F-bottom) by an indirect method based on competition of  
164 SOD with Water Soluble Tetrazolium salt (WST-1) for reduction of O<sub>2</sub><sup>-</sup>. Briefly, 20 $\mu$ L of  
165 sample solution and 200 $\mu$ L of WST-1 solution were added and gently mixed. The reaction  
166 was initiated by adding 20 $\mu$ L of xanthine oxydase (XO) and xanthine mix (enzyme solution),  
167 which form the superoxide anion used by SOD and WST-1 (SOD Assay kit, SIGMA  
168 ALDRICH). Two blanks were performed by replacing sample solution with milliQ-water  
169 (background reduction of O<sub>2</sub><sup>-</sup>).

170 After incubation (25°C for 20 min), the reduction of WST-1 by O<sub>2</sub><sup>-</sup> produces a yellowish  
171 formazan dye, the absorbance of which can be read at 450nm with POLARstar Omega  
172 microplate reader (BMG LABTECH). As the rate of reduction of WST-1 by O<sub>2</sub><sup>-</sup> is linearly  
173 related to XO activity and is inhibited by SOD, SOD activity is calculated as an inhibition  
174 activity of this reaction. Subsequently, a standard inhibition curve was performed using SOD  
175 from bovine erythrocytes (SIGMA ALDRICH). SOD activities were expressed in units per  
176 mg of protein (U/mg); 1 U of SOD being defined as the amount of enzyme inhibiting by 50%  
177 the reaction.

### 178 **2-7-Statistical analysis**

179 Correlation analysis between specific activities (PO and SOD) and protein contents were  
180 performed in order to assess the relation between those two variables. As we are interested in  
181 highlighting any monotonic relationship (either decreasing or increasing) and not only linear  
182 relationships, we used Spearman test of rank correlation.

183 Two-way split-plot analyses of variance (ANOVA) were conducted to determine differences  
184 in PO, SOD and protein contents according to the Injection Condition (fixed effect with three  
185 levels: SSW, LP2 and CECT4600<sup>T</sup>), Time p.i. (fixed effect with six levels: T0, 36 and 72



186 hours, 7, 14 and 30 days p.i.) and their interaction (Injection X Time p.i.). The unit of  
187 replication was the tank where injected clams receiving the same injection condition were  
188 placed (n=3 tanks for each injection condition). The main plots were injection levels (SSW,  
189 LP2 and CECT4600<sup>T</sup>), subplots were sampling times (time p.i.). Three-way split-split-plot  
190 ANOVAs were used to determine differences in PO, SOD and protein contents upon injection  
191 condition, time p.i. and tissue. Characteristics of this three-way split-split-plot were similar to  
192 those of the two-way split-plot analysis except that sub-subplots were performed using tissue  
193 levels (hemolymph, EFs and mantle). The model used for these analyses was the linear model  
194 using the aov function in R that fits an ANOVA model. Where significant differences were  
195 obtained, a post-hoc analysis was carried out using Tukey's HSD test to determine which  
196 means were significantly different for main effects and interaction effects. Normality and  
197 homoscedasticity of residuals were assessed by graphical methods. Explained variables were  
198 all transformed using the  $\log(x+1)$  transformation in order to satisfy normality and  
199 homoscedasticity assumptions.

200 All analyses were performed with R (version 2.15.3;) [39] and the "TukeyC" package [40].

201

### 202 3-Results

#### 203 3-1- Enzyme activities correlations with protein contents

204 Prior to further investigation, enzyme specific activities were plotted as a function of protein  
205 content (Fig.1A-D). Those representations associated with non-zero correlation coefficients  
206 demonstrate particular correlations between specific activities and protein contents. In the  
207 mantle, PO specific activities ( $\text{I.U.mg}^{-1}$ ) (Fig.1A) are negatively correlated with protein  
208 content ( $\rho = -0.98$ , p-value  $< 0.001$ ). In hemolymph, PO and SOD specific activities ( $\text{U.mg}^{-1}$ )  
209 (Fig.1B and D) are both negatively correlated with protein contents (respectively for PO and  
210 SOD,  $\rho = -0.52$ , p-value  $< 0.001$  and  $\rho = -0.29$ , p-value = 0.001). Yet, specific data do not  
211 truly represent PO and SOD activities as they superficially lower activities while protein  
212 content rises. Thus, PO volumic activities ( $\text{I.U.mL}^{-1}$ ) in the mantle and hemolymph, and SOD  
213 volumic activities ( $\text{U.mL}$ ) in hemolymph were preferentially used. Conversely, in the mantle,  
214 SOD specific activities (Fig.1.C) are positively correlated with protein contents ( $\rho = 0.43$ , p-  
215 value  $< 0.001$ ). This allows for the direct use of SOD specific activity in the mantle. These

216 results indicate that widely used specific activities are not always relevant, depending on the  
 217 considered tissue and enzyme.

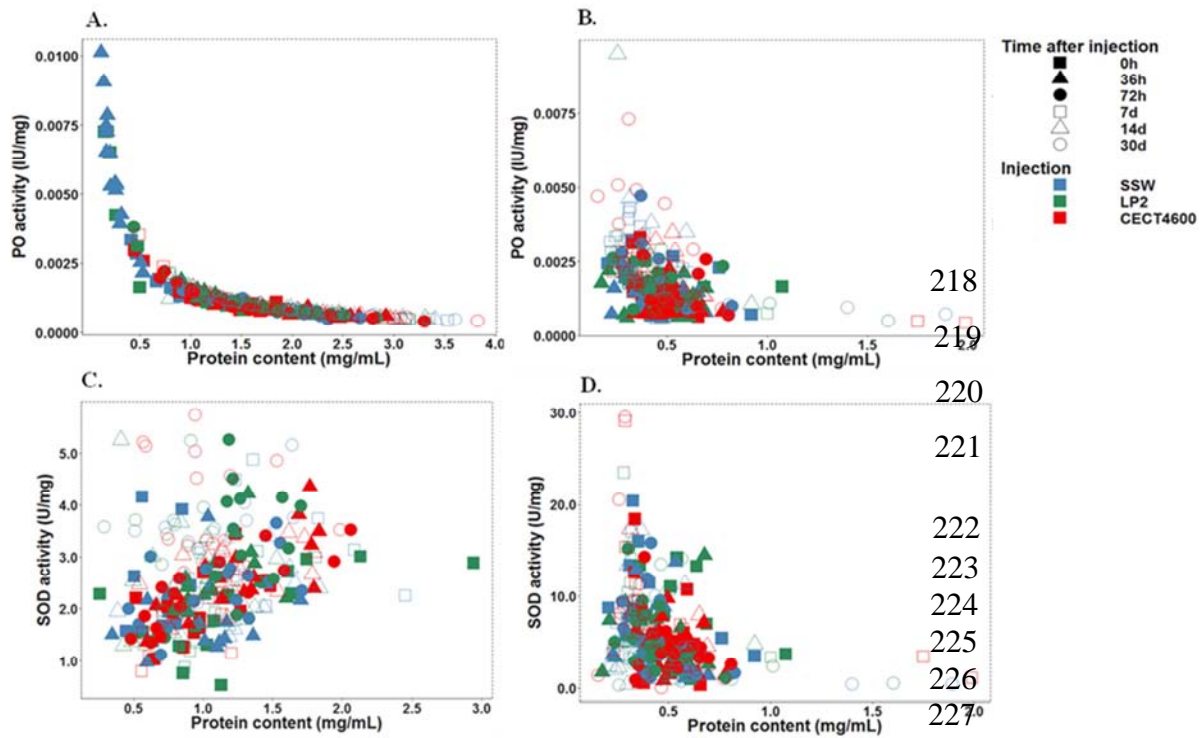


Fig. 1: PO specific activity and SOD specific activity as a function of protein content in the mantle (A. and C.) and in hemolymph (B. and D.) respectively. Note that axes do not cover the same variation range in all figures.

### 231 3-2 Protein contents and enzymatic activities

232 Results of statistical analyses are summarized in Tables 1, 2 and 3.

233 Protein contents varied as a function of injection  $\times$  tissue  $\times$  time interaction. In the mantle, a  
 234 significant time and injection interaction affected protein contents as it increased in  
 235 CECT4600<sup>T</sup>- and LP2-challenged clams 36 hours p.i (Fig.2A). In hemolymph, there was no  
 236 significant time and injection interaction but protein contents were higher in LP2-challenged  
 237 and control clams 30 days p.i. (Fig.2B). In extrapallial fluids, there was no significant

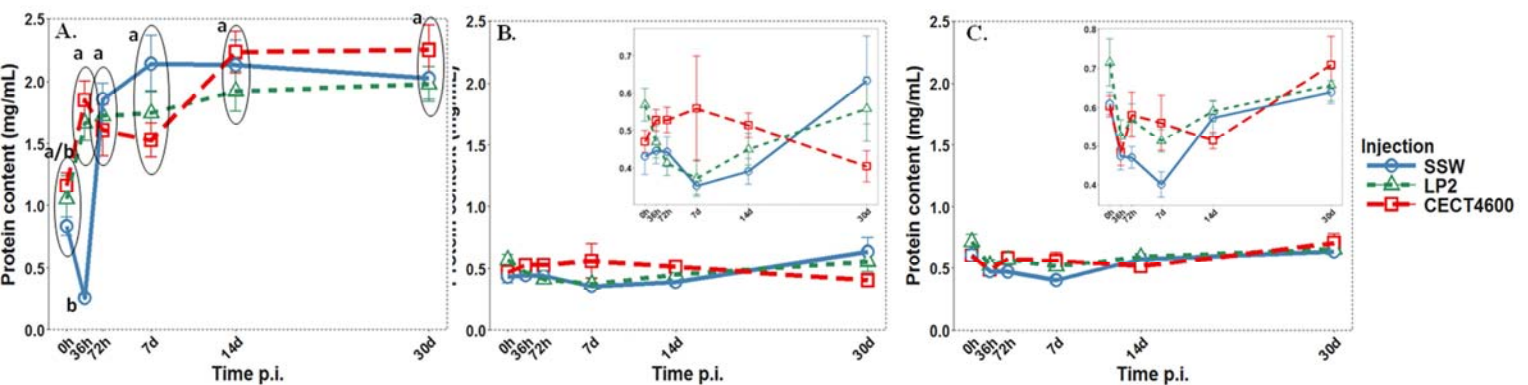
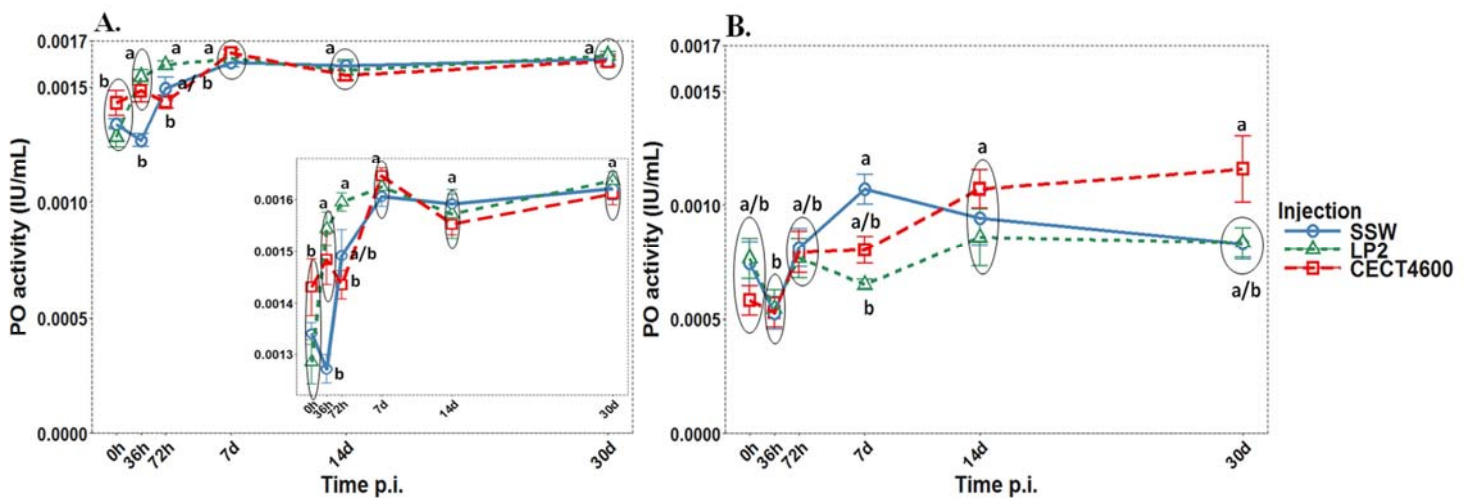


Fig. 2: Protein contents in the mantle (A), hemolymph (B with a zoom in Y value in the top right corner) and extrapallial fluids (C with a zoom in Y value in the top right corner) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600<sup>T</sup> and LP2) or Sterile Seawater (SSW). Error bars represent standard error. Letters indicate significant differences in protein contents upon injection between different groups and times (Split-plot ANOVA followed by post hoc Tukey's HSD test,  $\alpha = 0.05$ ). N = 15

238 interaction effect but significant first order effects of time and injection exist (Fig.2C).  
 239 PO activities also varied as a function of time  $\times$  injection  $\times$  tissue interaction. In the mantle,  
 240 PO is influenced by a significant time and injection interaction. 36 hours p.i. PO activity  
 241 increased in CECT4600<sup>T</sup>- and LP2-challenged clams. Additionally, three days p.i., PO  
 242 activity was higher in LP2-challenged clams than in CECT4600<sup>T</sup>-challenged and control  
 243 clams (Fig.3.A). In hemolymph, PO activities are lower in CECT4600<sup>T</sup>- and LP2-challenged  
 244 clams 7 days p.i. in comparison with control clams and are increased in CECT4600<sup>T</sup>-  
 245 challenged clams 30 days p.i. (significant time and injection interaction) (Fig.3B).



246 Fig. 3: PO activities in the mantle (A, with a zoom in Y value in the bottom right corner) and hemolymph (B) of *V. philippinarum*  
 247 post-injection (p.i.) with *V. tapetis* (strains CECT4600<sup>T</sup> and LP2) or Sterile Seawater (SSW). Error bars represent standard error.  
 248 Letters indicate significant differences in PO activity upon injection and between groups and times (Split-plot ANOVA followed  
 249 by post hoc Tukey's HSD test,  $\alpha = 0.05$ ). N = 15 for each sampling time and injection combination.

250  
 251 SOD activities varied as a function of time  $\times$  injection  $\times$  tissue interaction. In the mantle,  
 252 SOD activity increased in CECT4600<sup>T</sup> and LP2-challenged clams 36 hours p.i. and in  
 253 contrary, drops in the same clams 7 days p.i. (Fig.4A) (significant time and injection  
 254 interaction). In hemolymph, no significant effect was observed but a rise in SOD activity  
 255 occurred in CECT4600<sup>T</sup>-challenged clams 7 days p.i. (Fig.4A).

256  
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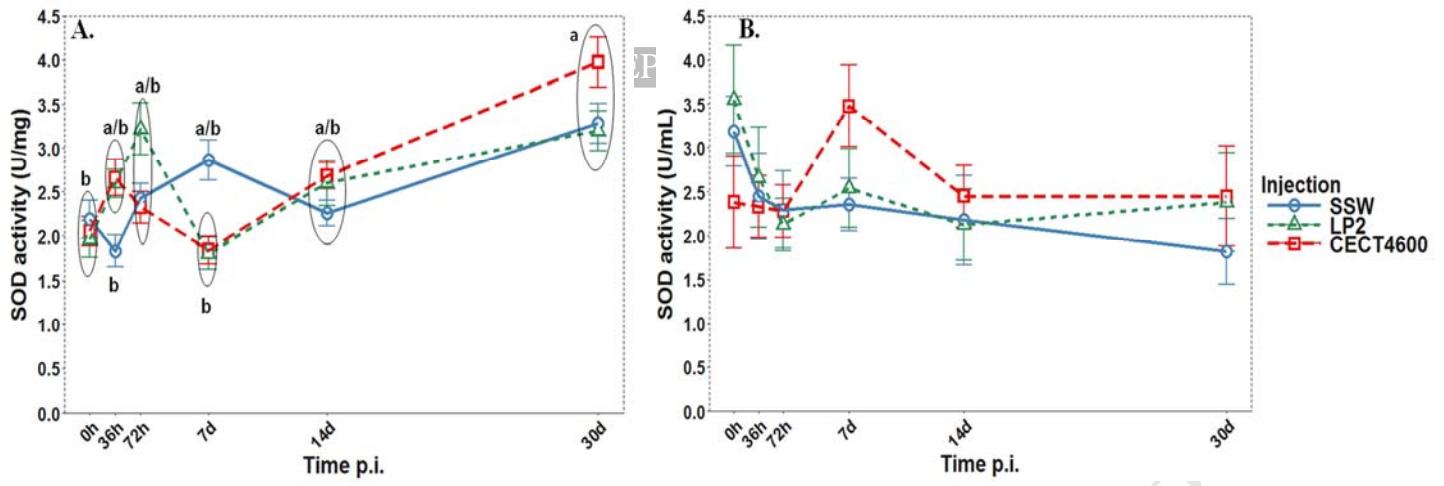


Fig. 4. SOD activities in the mantle (A) and hemolymph (B) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600<sup>T</sup> and LP2) or Sterile Sea Water (SSW). Error bars represent standard error. Different letters indicate significant differences in SOD activity upon injection between groups and times (Split-plot ANOVA followed by post hoc Tukey's HSD test,  $\alpha = 0.05$ ). N = 15 for each sampling time and injection combination.

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Table 1: Summary of the split-split plot three-way ANOVAs on the effect of injection condition, tissue sampled and time p.i. on protein contents, PO and SOD activity in the hemolymph of *V. philippinarum*

Source of variation	d.f.	Protein contents		PO activity		SOD activity	
		F		F		F	
<b>Main plot analysis</b>							
Injection	2	15,7	**	0,377	N.S.	2,607	N.S.
Error (tank)	6						
<b>Subplot analysis</b>							
Tissue	2	1601,553	***	268,97	***	7,649	**
Injection × tissue	4	4,934	*	2,015	N.S.	0,063	N.S.
Error (tank × injection × tissue)	12						
<b>Sub-subplot analysis</b>							
Time	5	13,005	***	12,266	***	0,242	N.S.
Injection × time	10	4,276	***	1,804	N.S.	1,821	N.S.
Tissue × time	10	12,707	***	10,248	***	5,591	***
Injection × tissue × time	20	5,209	***	3,279	***	2,584	*
Error (tank × injection × tissue × time)	90						

Independent variables were injection condition (SSW, CECT4600<sup>T</sup> and LP2) and time p.i. (0h, 36h, 72h, 7d, 14d and 30d).

Significant differences are indicated by \*\*\*:  $p < 0.001$ ; \*\*:  $0.001 < p < 0.01$ ; \*:  $0.01 < p < 0.05$ ; and no significant difference: N.S. (ANOVA,  $\alpha=0.05$ ).

267

268

Table 2: Summary of the split plot two-way ANOVAs on the effect of injection condition and time p.i. on protein contents, PO and SOD activity in the mantle of *V. philippinarum*

Source of variation	d.f.	Mantle					
		Protein contents		PO activity		SOD activity	
		F		F		F	
<b>Main plot analysis</b>							
Injection	2	14,59	**	4,098	N.S.	0,925	N.S.
Error (tank)	6						
<b>Subplot analysis</b>							
Time	5	15,035	***	18,825	***	13,039	***
Injection × time	10	5,758	***	2,963	*	3,763	**
Error (tank × injection × time)	30						

Independent variables were injection condition (SSW, CECT4600<sup>T</sup> and LP2) and time p.i. (0h, 36h, 72h, 7d, 14d and 30d).

Significant differences are indicated by \*\*\*:  $p < 0.001$ ; \*\*:  $0.001 < p < 0.01$ ; \*:  $0.01 < p < 0.05$ ; and no significant difference: N.S. (ANOVA,  $\alpha=0.05$ ).

269

Table 3: Summary of the split plot two-way ANOVAs on the effect of injection condition and time p.i. on protein contents, PO and SOD activity in the hemolymph of *V. philippinarum*

Source of variation	d.f.	Hemolymph					
		Protein contents		PO activity		SOD activity	
		F		F		F	
<b>Main plot analysis</b>							
Injection	2	1,376	N.S.	2,39	N.S.	0,332	N.S.
Error (tank)	6						
<b>Subplot analysis</b>							
Time	5	1,428	N.S.	12,462	***	2,114	N.S.
Injection × time	10	1,985	N.S.	2,869	*	1,04	N.S.

Error (tank × injection × time)

30

Independent variables were injection condition (SSW, CECT4600<sup>T</sup> and LP2) and time p.i. (0h, 36h, 72h, 7d, 14d and 30d).

Significant differences are indicated by \*\*\*:  $p < 0.001$ ; \*\*:  $0.001 < p < 0.01$ ; \*:  $0.01 < p < 0.05$ ; and no significant difference: N.S. (ANOVA,  $\alpha=0.05$ ).

## 271 4-Discussion

### 272 4-1 Time-inconsistency of the overall response in protein contents and enzymatic activities

273 The present study reveals complex interactions between the time p.i., the injection condition  
274 and the tissue considered. Indeed, kinetic responses of protein contents, PO and SOD  
275 activities do not highlight a consistent pattern. Protein contents do not vary significantly with  
276 injection and time p.i. in EFs and hemolymph serum while an increase, followed by  
277 stabilization, is observed 36h p.i. in the mantle. Yet, protein contents of fluids of *V.*  
278 *philippinarum* were previously analyzed after an experimental challenge with *V. tapetis* in the  
279 pallial cavity and results pointed to a significant decrease of protein concentrations in EFs and  
280 hemolymph [7, 16]. In our study, increases and decreases are both observed in SOD and PO  
281 activities depending on the tissue. Though these enzymes have never been monitored to our  
282 knowledge for such long periods of time in Manila clams challenged with *V. tapetis*.

283 Additionally, control individuals exhibited variations in protein contents, in PO and SOD  
284 activities over experiment, revealing a response to SSW injection. These shifts were the  
285 results from manipulation of the clam especially during experimental injection. The handling  
286 effect on BRD development has been previously study in the Manila clam [41]. In our study,  
287 clams were removed from their tank 12 hours prior to treatment and injection done through  
288 the periostracal lamina causing a little hole within it. This way, injections allowed in  
289 particular, entrance of pallial water and so dilution of the extrapallial compartment content  
290 and thus induced a stress response in addition to the injected liquid itself. In our study, control  
291 animals, also stressed by the injection itself, must be regarded as control with respect to a  
292 biotic stress which is the injection of bacteria. However, the shifts observed in these control  
293 animals should be kept in mind when considering the results for bacteria-injected animals.  
294 Finally, another factor contributing to the inconsistency of the overall response of protein  
295 concentrations and enzymatic activities in this study was the inter-individual variability,  
296 particularly observed in fluids. Notwithstanding these inconsistencies during the experiment,  
297 when all factors considered, significant interactions are highlighted and are consistent with  
298 previous studies.

#### 299 **4-2-Tissue-dependent response of enzymatic activities**

300 A main result of this study is the differential PO and SOD changes between tissues. Activities  
301 of these enzymes are tissue-dependent over all tested conditions (without infection and with  
302 infection by two different strains). PO activity was higher in the mantle than in the serum, and  
303 correlations between specific activities and protein contents were more marked for the mantle.  
304 Differences in pattern between PO activities in various tissues are also known in the pacific  
305 oyster, *Crassostrea gigas* [42].

306 In our study, correlations between SOD specific activities and protein contents in the mantle  
307 and in hemolymph serum suggest two different profiles of SOD-like activity in these tissues.

308 These results are coherent with the distribution of the different SOD types and thus, their  
309 putative different functions. In fact, there are three main types of SOD: intracellular cytosolic  
310 Cu/Zn-SOD (ic Cu/Zn-SOD), intracellular mitochondrial Mn-SOD (ic Mn-SOD) and  
311 extracellular Cu/Zn-SOD (EC-SOD) [43]. EC-SOD, which is the only SOD type present in  
312 serum, could function differently from other SOD types present in the mantle in terms of  
313 mechanism. To date, only ic Cu/Zn-SOD and ic Mn-SOD genes have been identified and  
314 characterized in hemocytes and in the gills of *V. philippinarum* [33, 34]. Umasuthan and  
315 collaborators [34] highlighted differential expression of the two SOD genes in terms of tissue  
316 and in terms of SOD-type. Attempts to purify and characterize EC-SOD in bivalves were  
317 performed but failed to find SOD activity associated with the purified proteins. Nevertheless,  
318 few studies reported EC-SOD activity in bivalves. Immunoblot analyses of intracellular and  
319 extracellular Cu/Zn-SODs showed that when the clam, *Chamelea gallina* was exposed to  
320 anoxia or salinity stresses, EC-SOD in hemolymph was induced, whereas ic Cu/Zn-SOD  
321 activity was reduced in hemocytes [44, 45]. These findings are in concordance with different  
322 activity profiles of SODs depending on the considered tissue.

323

#### 324 **4-3 Time- and tissue-dependent response of protein contents and enzymatic activities**

##### 325 **4-3-1 A response governed by BRD development**

326 Another factor governing PO and SOD activity variations in our study was p.i. time. Results  
327 underlined an early response in the mantle and a later one in hemolymph that can be linked  
328 with BRD developmental stages. In natural conditions, the entry of the pathogen into the  
329 pallial space and the colonization of the periostracal lamina provokes alterations in

330 periostracum secretions and disruption of the lamina, permitting *V. tapetis* to penetrate in the  
331 Peripheral Extrapallial Fluids (PEFs) [46]. The normal deposition of periostracal lamina is  
332 seriously affected, resulting in a characteristic brown conchyolin deposit a few days after  
333 challenge with *V. tapetis* in the pallial fluids [4]. If the brown deposit leads to the occurrence  
334 of lesions, the bacteria penetrate the external epithelium of the mantle and proliferate in the  
335 tissues [4, 47]. In our study, the injection was done directly in the peripheral extrapallial  
336 cavity (between the mantle and the shell), thus accelerating BRD development by bypassing  
337 the periostracal lamina. As this physical barrier was crossed, *V. tapetis* (strains CECT4600<sup>T</sup>  
338 and LP2) was “helped” during the colonization phase in the extrapallial fluids (EFs). This  
339 could explain the response observed in the mantle at early times as this tissue is directly in  
340 contact with EFs. Maes [48] showed that *V. tapetis* inoculation in the clam pallial cavity  
341 induced faster mortalities than balneation exposure as the first physical barrier was ignored.  
342 More recently, Allam *et al.* [47] explored the role of the site of *V. tapetis* injection in Manila  
343 clam on BRD development. They found that mortalities reached 100% with animals injected  
344 in the adductor muscle and the central extrapallial space after making a hole in the shell (12  
345 and 14days p.i. respectively) and highlighted the role of epithelium and pallial muscle  
346 attachment as external barriers. In our study, the concentration of *V. tapetis* used for  
347 inoculation was lower than that in Allam *et al.*'s study [47] ( $5.10^7$  UFC/individual) and no  
348 damage was done to the shell due to extrapallial injections, explaining our low rates of  
349 mortalities (1.33% over the 30 days of experiment, data not shown). Previous work  
350 demonstrated two key moments in the general immune response of Manila clam to *V. tapetis*  
351 pallial cavity injection, a first response within 24 hours p.i. and a second response at later  
352 times, 7 to 14 days p.i. [16-18].

#### 353 ***4-3-2 An early response associated with cellular factors in the mantle***

354 In the present study, protein contents, PO and SOD activities rise in the mantle between T0  
355 and 72h p.i. in CECT4600<sup>T</sup> and LP2 injected clams. These rises could be explained by  
356 synthesis or release of proteins in infected zone to counter bacteria. PO activity in hemolymph  
357 cellular fraction of *C. gigas* has been shown to exhibit antibacterial activities against *Vibrios*  
358 shortly after challenges (between 0h and 7h p.i.) [49]. Additionally, Umasuthan and  
359 collaborators [34, 50] pointed out the involvement of *V. philippinarum* antioxidant enzymes



360 in the antibacterial response against *V. tapetis* by showing the time-dependence of this  
361 response. Moreover, after bacterial challenge, increase in SODs expression was observed  
362 within two days p.i. in bivalves and may be associated with scavenging ROS induced by  
363 bacteria [33, 34, 50, 51].

364 The early time responses observed in our study were previously detected in hemocytes of  
365 infected clams, resulting in an increase in total hemocytes count (THC) in the hemolymph and  
366 EFs [17, 18, 47]. In addition, Allam *et al.* [47] also observed a rise in phagocytic rates in EFs  
367 between 30 minutes and two hours p.i. when clams were injected in the extrapallial space with  
368 CTC-labeled *V. tapetis*. The enzymatic study of leucine-aminopeptidase (LAP) activity in  
369 hemolymph of *V. tapetis*-challenged clams showed similar results: a significant increase three  
370 days p.i. in the cellular fraction [17].

371 Taken together, these results suggest a first response at early times that is mainly cellular;  
372 high enzymatic levels in hemolymph cellular fractions were interpreted as an increase of  
373 enzyme synthesis in activated cells, triggered by *V. tapetis* injection [17].

#### 374 ***4-3-3 A later response associated with humoral factors in hemolymph and extrapallial fluids***

375 A later response was observed in our study, consisting of higher protein contents in the  
376 acellular fraction of hemolymph and EFs in *V. tapetis* injected-clams. Indeed, in hemolymph,  
377 protein concentrations were higher in CECT4600<sup>T</sup>-injected clams between 3 and 14 days p.i.,  
378 in EFs they were higher in both CECT4600<sup>T</sup>- and LP2-injected clams 7 days p.i.. These  
379 results are different from those observed by Allam & Ford [14] who found increased protein  
380 concentrations in clams injected in the central extrapallial space three days p.i., irrespective of  
381 the treatment (*i.e.*, bacteria or SSW). According to the authors, this augmentation was  
382 associated with the shell damage repair as injection was performed by making a hole in the  
383 central part of the shell [14]. In our study, peripheral extrapallial injection was done by  
384 inserting a needle between mantle and shell so that no damage occurred on the shell,  
385 explaining differences with the Allam & Ford study. Augmentation of protein contents in  
386 fluids at later times was observed only in bacteria-injected clams, suggesting the synthesis or  
387 secretion of enzymes and peptides in extracellular compartments in order to counteract the  
388 bacteria [17]. Between 0 and 36 hours p.i. protein contents in EFs decreased in both bacterial  
389 and SSW injections. This result underlined a 'dilution-effect' post-injection as SSW or  
390 bacteria solution was added to the EFs during injection.

391 In our study, PO activity in hemolymph supernatant rose between 7 and 14 days p.i. in  
392 CECT4600<sup>T</sup>- and LP2-injected clams and then stagnated until the end of experiment.  
393 Presence of bacterial products, such as lipopolysaccharides or peptidoglycans, lead to  
394 degranulation of semigranular and granular hemocytes containing proPO (the inactive form of  
395 PO) [25]. After degranulation in the hemolymph, proPO is activated by proteolytic cleavage  
396 by serine proteases, yielding active PO [22]. Thus the increase of PO activity in acellular  
397 fraction we observed at later times could be associated with hemocyte degranulation induced  
398 by *V. tapetis* challenge. This is also consistent with the results of Allam & Ford [14] who  
399 established a loss of granules in hemocytes after *V. tapetis* challenge in *V. philippinarum*.

400 In the present study, SOD activity in hemolymph supernatant rose to its maximum at day 7  
401 p.i. in CECT4600<sup>T</sup>-injected clams and then decreased until the end of the experiment. EC-  
402 SOD, is produced in hemocytes and secreted into extracellular fluids, where it represents the  
403 major SOD-isoenzyme [52]. An EC-SOD gene was characterized in the bay scallop,  
404 *Argopecten irradians*, and its expression in hemocytes was studied during the 48 hours  
405 following a *Vibrio anguillarum* challenge [53]. The time-dependence of EC-SOD expression  
406 in hemocytes was established with a peak of gene-expression at early times (12 hours p.i.) but  
407 its activity in serum was not assessed. This difference in kinetic response of EC-SOD activity  
408 and the expression of its associated gene might be due to several factors such as post-  
409 transcriptional regulation, putative activation of the inactive form of EC-SOD or excretion of  
410 it into the extracellular compartment [43]. To our knowledge, this is the first account of EC-  
411 SOD activity in the acellular fraction of hemolymph in a bivalve challenged with *Vibrio sp.*,  
412 and in order to confirm this hypothesis, it would be interesting to analyze gene expression of  
413 SOD and PO.

414 In previous work, responses observed at later times in *V. philippinarum* challenged with *V.*  
415 *tapetis* resulted in THC rising to its maximum at 7-14 days p.i. and decreasing after 14 days  
416 p.i. [16, 17]. Additionally, LAP activities in the cellular fraction of hemolymph reached their  
417 peak 7 days p.i. [17] and lysozyme activity in this same fraction was significantly higher in  
418 infected clams 14 and 28 days p.i. [16].

419 More generally, as hemolymph is a circulating fluid, the response observed at later times may  
420 be explained by an “integration time” needed by the organism to counteract the infection and  
421 also by the fact that the response is diluted across the whole organism.

422

#### 423 **4-4 Pathogenicity of *V. tapetis***

424 Our results suggest two different time- and tissue-dependent responses toward injections: a  
425 non-specific response related to a “bacteria-effect” and a specific response associated with  
426 CECT4600<sup>T</sup>'s higher pathogenicity. The “bacteria-effect” or non-specific response was  
427 mainly observed in tissues that were directly injected, i.e. the mantle and EFs. Protein  
428 contents, PO and SOD activities were significantly higher in CECT4600<sup>T</sup>- and LP2-  
429 challenged clams 36 hours p.i. in mantle and 7 days p.i. in EFs. These higher protein  
430 concentrations are a result of a synthesis and/or release of proteins in the affected tissues in  
431 order to counteract the bacteria.

432 In the mantle, PO and SOD activities were significantly higher in LP2-challenged clams than  
433 in CECT4600<sup>T</sup>- and SSW-injected clams three days p.i. In hemolymph, protein contents, PO  
434 and SOD activity were higher in the case of CECT4600<sup>T</sup>-injection, 3-14 days p.i., 14 days p.i.  
435 and 7 days p.i. respectively. These specific biochemical responses point to the conclusion that  
436 there are differences between pathogenicities of the two *V. tapetis* strains. This is also in  
437 accordance with BRD stages (Conchyolin Deposit Stages) determined in clams 30 days p.i. as  
438 almost all CECT4600<sup>T</sup>-challenged individuals were symptomatic whereas 25% of LP2-  
439 challenged ones were still asymptomatic (data not shown). Additionally, the most advanced  
440 symptoms were observed in CECT4600<sup>T</sup>-challenged clams and lower stages were found in  
441 LP2-challenged ones (data not shown).

442 To infect organisms and proliferate, Vibrios have to counter host defenses by different means  
443 including phagocytosis inhibition, oxidative burst prevention and deregulation of signaling  
444 pathways [54, 55]. In this single host experiment, interactions between host immune defenses  
445 and pathogen virulence factors depended on Vibrios pathogenicity [56]. This is why two *V.*  
446 *tapetis* strains were injected in *V. philippinarum* in this study: CECT4600<sup>T</sup>, isolated from  
447 BRD-affected *V. philippinarum* [5, 57], and LP2, isolated from *Symphodus melops* in 1999 in  
448 Norway [10]. The latter was previously considered as a non-pathogenic strain to Manila clam.  
449 *V. tapetis* possess several virulence factors that could explain the pathogenicity of the two  
450 strains used in this study. For example, adhesion to cells/tissues is accomplished through the  
451 presence of pili in *V. tapetis* [5, 57] while the presence of smooth lipopolysaccharides  
452 facilitates entry of the bacteria into the host's cells and tissues helping it avoid phagocytosis  
453 [47]. Pathogenicity of *V. tapetis*, mainly studied in Manila clam hemocytes, results in a

454 number of changes to clam cells, including cell rounding, vacuolizations and loss of  
455 adherence capacity [12, 14]. Lopez-Cortez *et al.* [58] established that clam phagocytic activity  
456 was independent from pathogenicity degrees and host-range specificity of *V. tapetis* strain :  
457 soluble hemolymph proteins are not required for identification and internalization of the  
458 bacteria. These results support the non-specificity of immune responses toward the different  
459 strains we used in this study. Moreover, Choquet *et al.* [12] compared different strains of *V.*  
460 *tapetis* and classified LP2 strain as having a relatively low cytotoxic activity, whereas  
461 CECT4600<sup>T</sup> strain is highly cytotoxic. This is consistent with our results and explains the  
462 specific response obtained toward the two strains tested. However, toxicity mechanisms  
463 remain poorly understood even if some factors have been identified.

## 464 **Conclusion**

465 This study highlights the modulation of two enzymes involved in immune response of *V.*  
466 *philippinarum* following bacterial infection in the extrapallial cavity, phenoloxidase and  
467 superoxide dismutase. The bacteria *V. tapetis* induces a time- and tissue-dependent response  
468 in the Manila clam. First, an early response occurs in the mantle, close to the injection site.  
469 This first response is associated with an increase of protein contents, as well as PO and SOD  
470 activity. Afterwards, a later response follows in the clams' fluids, resulting in an increase of  
471 PO activities and a decrease of SOD activities. These two times in immune response were  
472 also observed in previous work focusing on cellular and humoral components of *V.*  
473 *philippinarum* injected in the pallial cavity with *V. tapetis* [16-18]. Another main result of this  
474 study is the difference of pathogenicity between the two *V. tapetis* strains characterized by  
475 higher levels of enzymes' activities in hemolymph (between 7 and 30 days) and lower levels  
476 in the mantle (72h p.i.) in CECT4600<sup>T</sup>-injected clams. This work clearly illustrates the host-  
477 pathogen interaction and its consequences for these two particular enzymes in fixed  
478 environmental conditions. This interaction can be modulated by environmental factors,  
479 particularly temperature [59-61] and further studies should focus on the impact of  
480 environmental conditions on the Manila clam's response to *V. tapetis* and the pathogen's  
481 virulence.

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