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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

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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.

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

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^T) 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^T 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^T (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 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⁻¹) = ($\Delta A \cdot \text{min}^{-1} \times \text{dilution factor}$) / 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 O₂⁻. Briefly, 20 μ L of
165 sample solution and 200 μ L of WST-1 solution were added and gently mixed. The reaction
166 was initiated by adding 20 μ 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 O₂⁻).

170 After incubation (25°C for 20 min), the reduction of WST-1 by O₂⁻ 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 O₂⁻ 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^T), 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^T), 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 (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 (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 (I.U.mL^{-1}) in the mantle and hemolymph, and SOD
213 volumic activities (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.

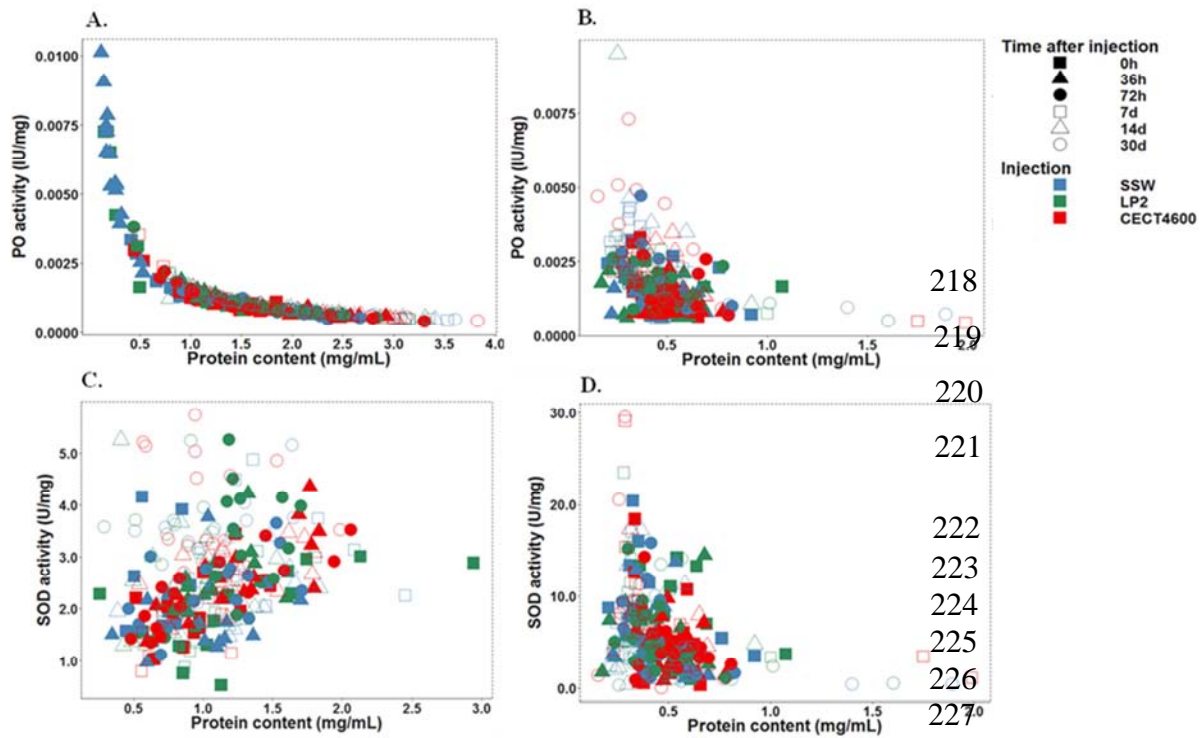


Fig. 1: PO specific activity and SOD specific activity as a function of protein content in the mantle (A. and C.) and in hemolymph (B. and D.) respectively. Note that axes do not cover the same variation range in all figures.

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^T- 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

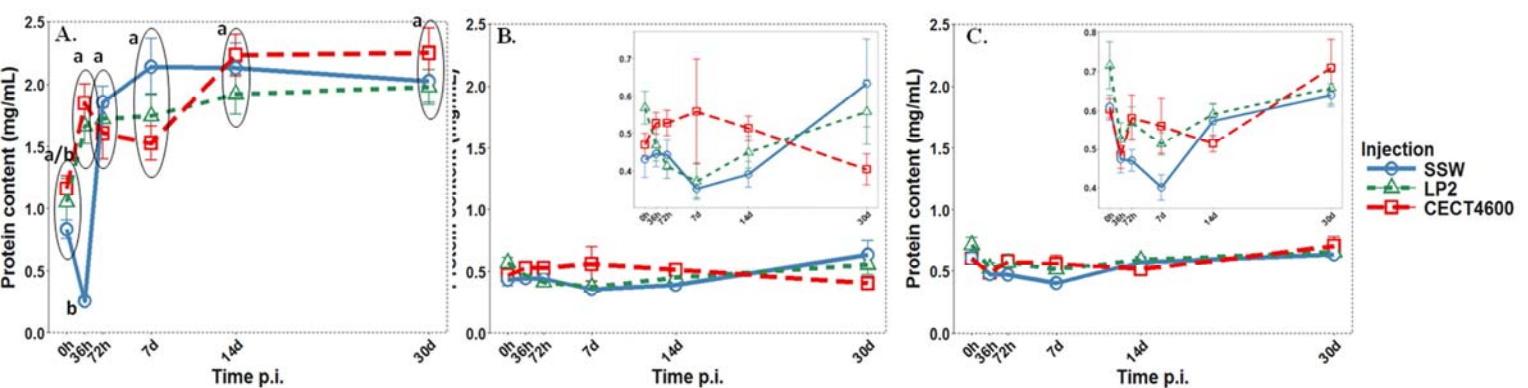
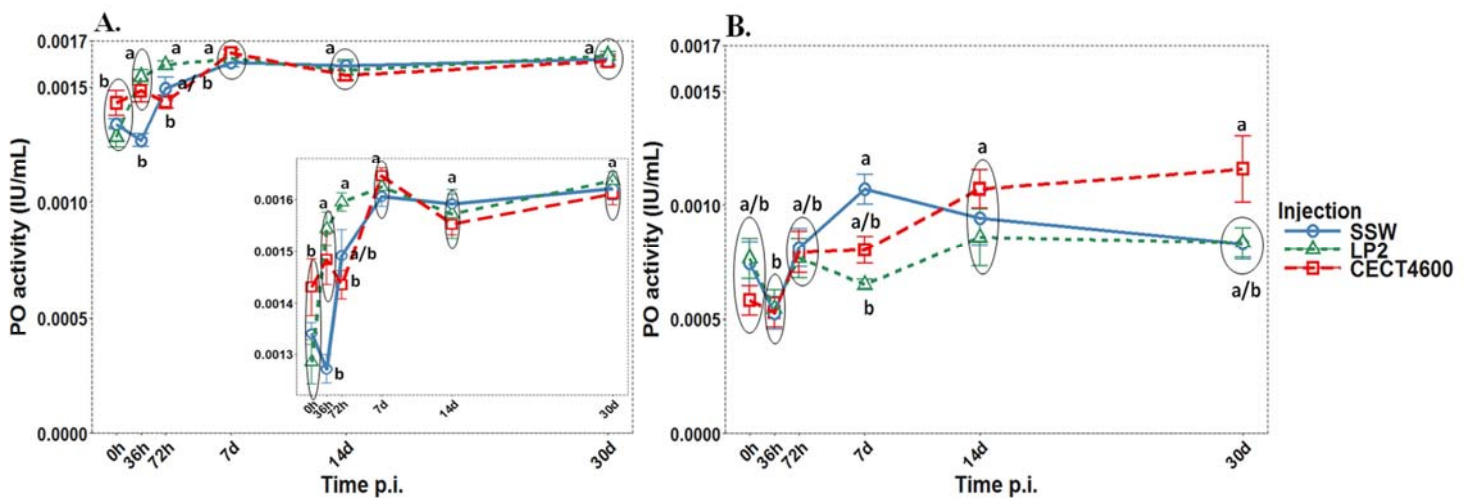


Fig. 2: Protein contents in the mantle (A), hemolymph (B with a zoom in Y value in the top right corner) and extrapallial fluids (C with a zoom in Y value in the top right corner) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600^T and LP2) or Sterile Seawater (SSW). Error 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^T- and LP2-challenged clams. Additionally, three days p.i., PO
 242 activity was higher in LP2-challenged clams than in CECT4600^T-challenged and control
 243 clams (Fig.3.A). In hemolymph, PO activities are lower in CECT4600^T- and LP2-challenged
 244 clams 7 days p.i. in comparison with control clams and are increased in CECT4600^T-
 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^T 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^T 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^T-challenged clams 7 days p.i. (Fig.4A).

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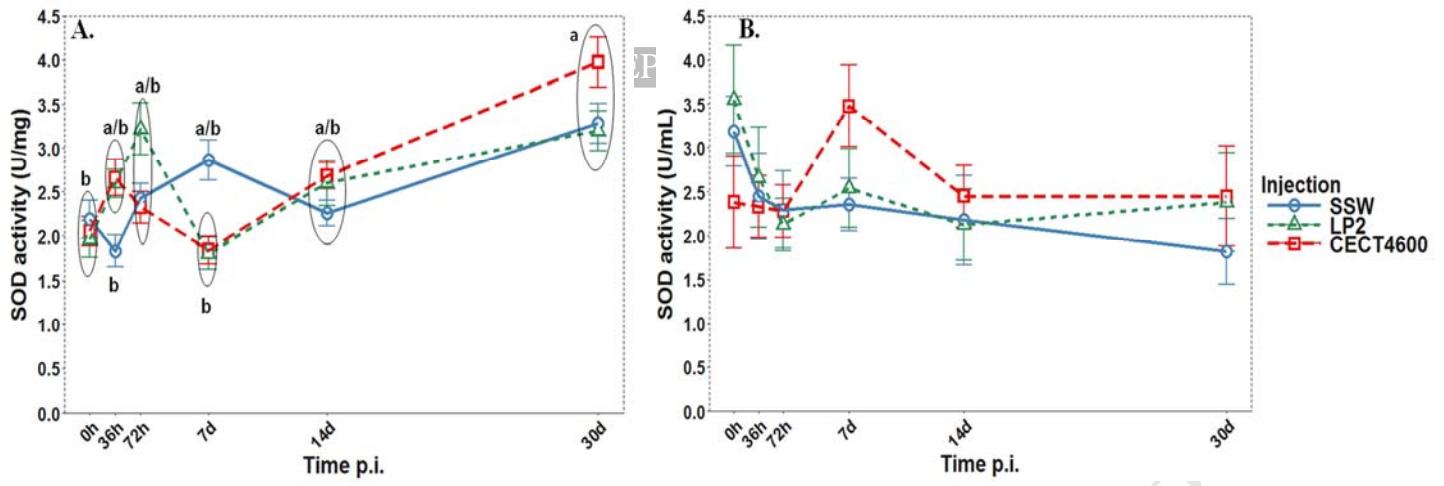


Fig. 4. SOD activities in the mantle (A) and hemolymph (B) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600^T and LP2) or Sterile Sea Water (SSW). Error bars represent standard error. Different letters indicate significant differences in SOD activity upon injection between 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	
Main plot analysis							
Injection	2	15,7	**	0,377	N.S.	2,607	N.S.
Error (tank)	6						
Subplot analysis							
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						
Sub-subplot analysis							
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^T 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	
Main plot analysis							
Injection	2	14,59	**	4,098	N.S.	0,925	N.S.
Error (tank)	6						
Subplot analysis							
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^T 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	
Main plot analysis							
Injection	2	1,376	N.S.	2,39	N.S.	0,332	N.S.
Error (tank)	6						
Subplot analysis							
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^T 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.

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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^T
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^T 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^T-injected clams between 3 and 14 days p.i.,
378 in EFs they were higher in both CECT4600^T- 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^T- 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^T-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.

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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^T'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^T- 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^T- and SSW-injected clams three days p.i. In hemolymph, protein contents, PO
434 and SOD activity were higher in the case of CECT4600^T-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^T-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^T-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^T, 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^T 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^T-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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