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Cédric Le Bris, Gaëlle Richard, Christine Paillard, Christophe Lambert, Catherine Seguineau, Olivier Gauthier, Fabrice Pernet, Fabienne Guérard

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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[1, *], Richard Gaelle [1], Paillard Christine [1], Lambert Christophe [1], Seguineau Catherine [1], Gauthier Olivier [1], Pernet Fabrice [2], Guerard Fabienne [1]

[1] 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
[2] 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

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

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

activity level and the level of infection by *Marteilia sydneyi* which is responsible for QX disease [26]. Indeed, a QX resistance breeding program developed along the Australian East coast has selected oysters with enhanced PO activity [27]. PO activity in *V. philippinarum* hemolymph has been biochemically characterized [28, 29] but variations in PO after infection has, to date, never been followed. Superoxide dismutase (SOD) (E.C. 1.15.1.1), like PO, is an oxidoreductase that contributes towards innate immunity by preventing the accumulation of Reactive Oxygen Species (ROS) in the organism, particularly superoxide anions (O_2^-). In marine bivalves, ROS are generated in larger proportions as the organism is exposed to biotic challenges such as *Vibrio* extracellular products [30, 31]. ROS are known to exhibit antimicrobial properties but overconcentration can lead to cellular damages: the antioxidant paradox [32]. In order to control the cell redox status, antioxidant enzymes are recruited to diminish ROS concentration. Intracellular SOD gene expression analyses performed in Manila clams infected with *Vibrio* species (*V. anguillarum*, *V. tapetis*) showed time-dependent variations over short periods (96 hours maximum) [33, 34], suggesting the regulation of this protein and its involvement in innate immunity of *V. philippinarum*. Nevertheless, SOD has not been studied biochemically in *Vibrio*-challenged clams so far, and it is interesting to focus on biological activities of PO and SOD over longer periods (up to 30 days after injection) since BRD symptoms require time to develop after infection.

Thus, the aim of this study was to investigate the effects *V. tapetis* infection on immune enzymatic activities in *V. philippinarum*. Clams were infected with two different *V. tapetis* strains and monitored for 30 days after infection. More precisely, our study compared PO and SOD immune activity, in different compartments, of clams injected with two different *V. tapetis* strains, considered more or less virulent, or with Sterile Sea Water (SSW) as a control.

2-Materials and methods

2-1-Biological material and acclimation procedure

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

2-2-Bacterial strains

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

2-3-Experimental injections

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

2-4-Fluid and tissue collection

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

2-4-1- Hemolymph and extrapallial fluid collection

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

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

2-4-2-Tissue collection and protein extraction

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

2-5-Protein determination

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

2-6-Enzyme activity assays

2-6-1-Phenoloxidase assays

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

PO specific activity (U.I.mg protein^{-1}) = (ΔA.min^{-1} × dilution factor) / Total protein concentration

Where ΔA.min^{-1} is the value of the increment of absorbance per minute, dilution factor is the sample's factor of dilution, and total protein concentration is the sample's protein concentration.

2-6-2-Superoxide dismutase assays

Total superoxide dismutase (SOD: EC 1.15.1.1) activity was assayed spectrophotometrically in 96-well microplates (Greiner 96-F-bottom) by an indirect method based on competition of SOD with Water Soluble Tetrazolium salt (WST-1) for reduction of O_2^-. Briefly, 20µL of sample solution and 200µL of WST-1 solution were added and gently mixed. The reaction was initiated by adding 20µL of xanthine oxydase (XO) and xanthine mix (enzyme solution), which form the superoxide anion used by SOD and WST-1 (SOD Assay kit, SIGMA ALDRICH). Two blanks were performed by replacing sample solution with milliQ-water (background reduction of O_2^-).

After incubation (25°C for 20 min), the reduction of WST-1 by O_2^- produces a yellowish formazan dye, the absorbance of which can be read at 450nm with POLARstar Omega microplate reader (BMG LABTECH). As the rate of reduction of WST-1 by O_2^- is linearly related to XO activity and is inhibited by SOD, SOD activity is calculated as an inhibition activity of this reaction. Subsequently, a standard inhibition curve was performed using SOD from bovine erythrocytes (SIGMA ALDRICH). SOD activities were expressed in units per mg of protein (U/mg); 1 U of SOD being defined as the amount of enzyme inhibiting by 50% the reaction.

2-7-Statistical analysis

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

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

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

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

3-Results

3-1- Enzyme activities correlations with protein contents

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

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

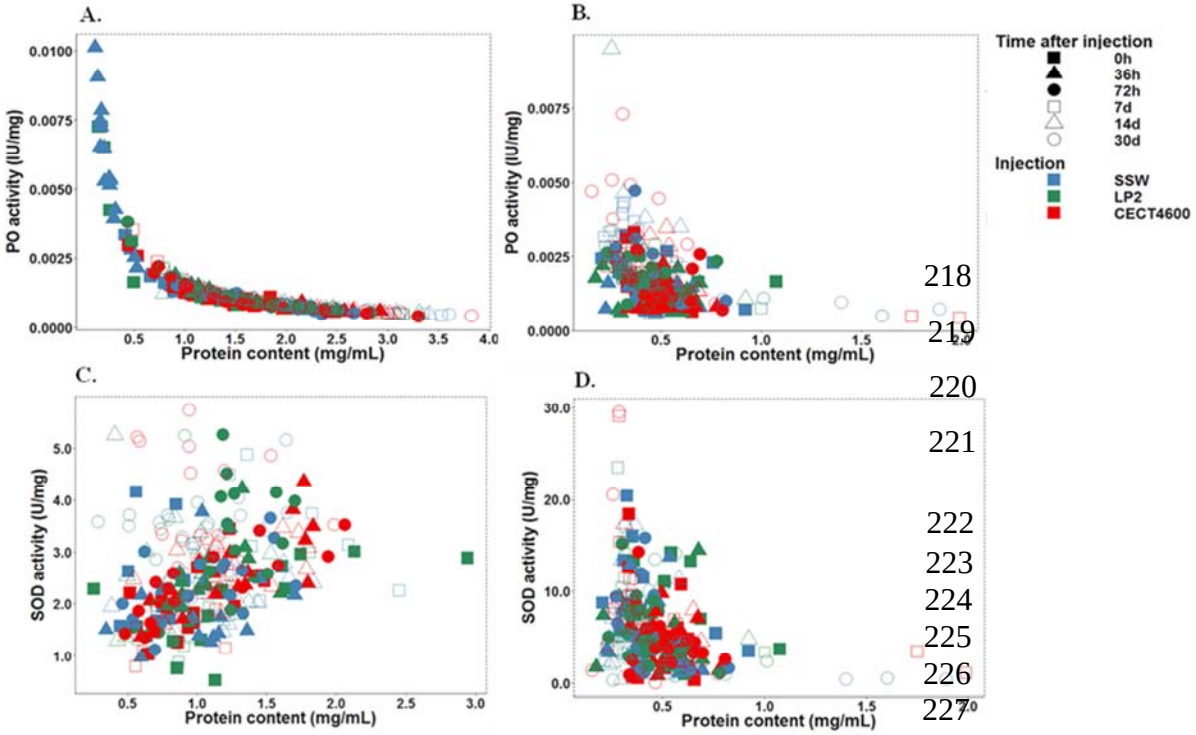

Fig. 1: PO specific activity and SOD specific activity as a function of protein content in the mantle (A. and C.) and in hemolymph (B. and D.) respectively. Note that axes do not cover the same variation range in all figures.

3-2 Protein contents and enzymatic activities

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

Protein contents varied as a function of injection × tissue × time interaction. In the mantle, a significant time and injection interaction affected protein contents as it increased in CECT4600^{T}- and LP2-challenged clams 36 hours p.i (Fig.2A). In hemolymph, there was no significant time and injection interaction but protein contents were higher in LP2-challenged and control clams 30 days p.i. (Fig.2B). In extrapallial fluids, there was no significant

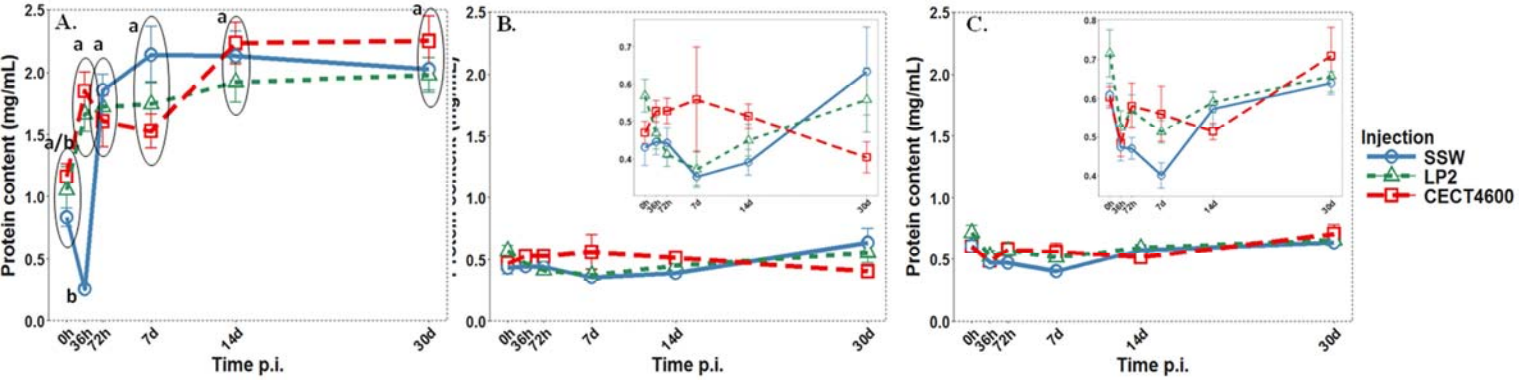

Fig. 2: Protein contents in the mantle (A), hemolymph (B with a zoom in Y value in the top right corner) and extrapallial fluids (C with a zoom in Y value in the top right corner) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600^{T} and LP2) or Sterile Seawater (SSW). Error 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, α = 0.05). N =15

interaction effect but significant first order effects of time and injection exist (Fig.2C). PO activities also varied as a function of time × injection × tissue interaction. In the mantle, PO is influenced by a significant time and injection interaction. 36 hours p.i. PO activity increased in CECT4600^{T}- and LP2-challenged clams. Additionally, three days p.i., PO activity was higher in LP2-challenged clams than in CECT4600^{T}-challenged and control clams (Fig.3.A). In hemolymph, PO activities are lower in CECT4600^{T}- and LP2-challenged clams 7 days p.i. in comparison with control clams and are increased in CECT4600^{T}-challenged clams 30 days p.i. (significant time and injection interaction) (Fig.3B).

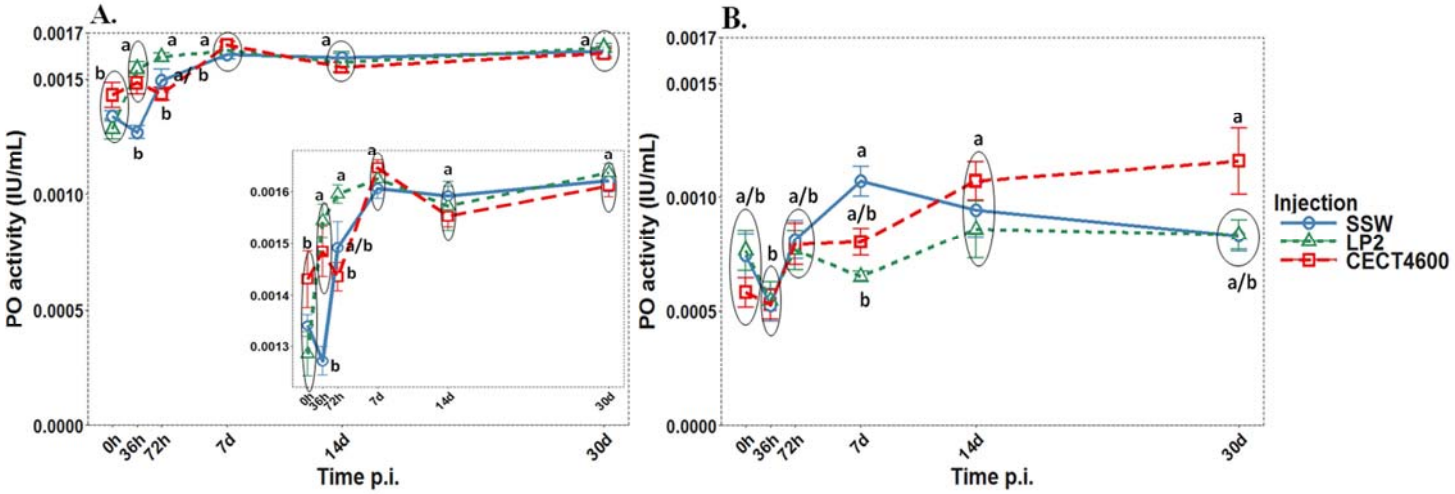

Fig. 3: PO activities in the mantle (A, with a zoom in Y value in the bottom right corner) and hemolymph (B) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600^{T} and LP2) or Sterile Seawater (SSW). Error bars represent standard error. Letters indicate significant differences in PO activity upon injection and 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.

SOD activities varied as a function of time × injection × tissue interaction. In the mantle, SOD activity increased in CECT4600^{T} and LP2-challenged clams 36 hours p.i. and in contrary, drops in the same clams 7 days p.i. (Fig.4A) (significant time and injection interaction). In hemolymph, no significant effect was observed but a rise in SOD activity occurred in CECT4600^{T}-challenged clams 7 days p.i. (Fig.4A).

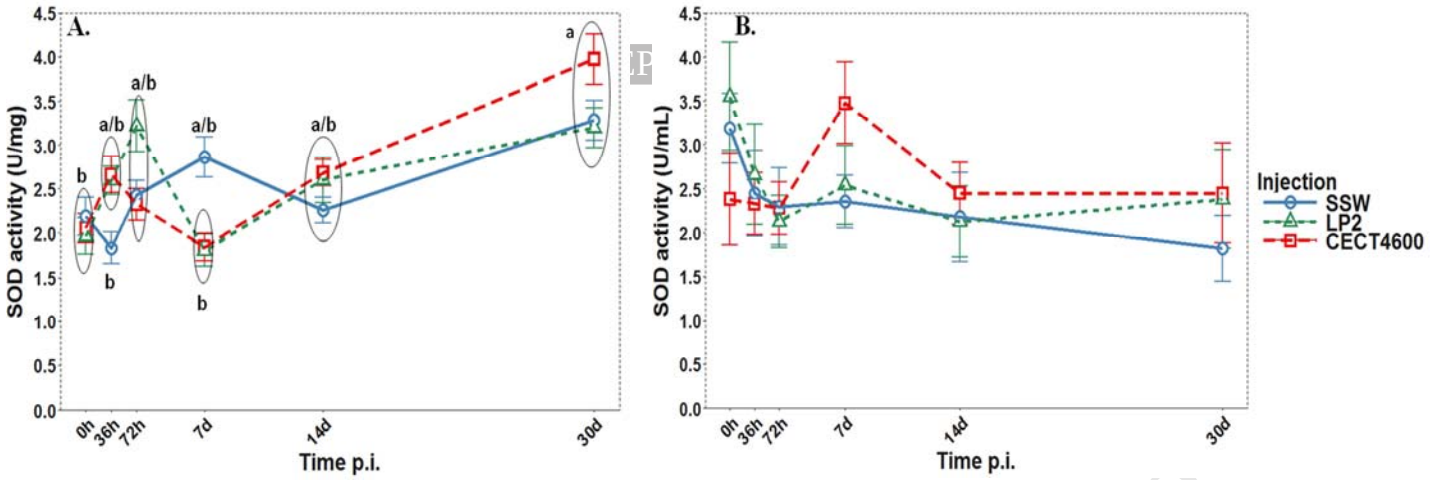

Fig. 4: SOD activities in the mantle (A) and hemolymph (B) of *V. philippinarum* post-injection (p.i.) with *V. tapetis* (strains CECT4600^T and LP2) or Sterile Sea Water (SSW). Error bars represent standard error. Different letters indicate significant differences in SOD activity upon injection between 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.

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*

		Protein contents		PO activity		SOD activity	
Source of variation	d.f.	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, $\alpha=0.05$).

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*

Mantle							
		Protein contents		PO activity		SOD activity	
Source of variation	d.f.	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, $\alpha=0.05$).

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*

Hemolymph							
		Protein contents		PO activity		SOD activity	
Source of variation	d.f.	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, $\alpha=0.05$).

4-Discussion

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

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

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

4-2-Tissue-dependent response of enzymatic activities

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

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

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

4-3-1 A response governed by BRD development

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

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

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

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

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

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

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

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

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

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

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

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

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

4-4 Pathogenicity of *V. tapetis*

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

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

To infect organisms and proliferate, Vibrios have to counter host defenses by different means including phagocytosis inhibition, oxidative burst prevention and deregulation of signaling pathways [54, 55]. In this single host experiment, interactions between host immune defenses and pathogen virulence factors depended on Vibrios pathogenicity [56]. This is why two *V. tapetis* strains were injected in *V. philippinarum* in this study: CECT4600^T, isolated from BRD-affected *V. philippinarum* [5, 57], and LP2, isolated from *Symphodus melops* in 1999 in Norway [10]. The latter was previously considered as a non-pathogenic strain to Manila clam.

V. tapetis possess several virulence factors that could explain the pathogenicity of the two strains used in this study. For example, adhesion to cells/tissues is accomplished through the presence of pili in *V. tapetis* [5, 57] while the presence of smooth lipopolysaccharides facilitates entry of the bacteria into the host's cells and tissues helping it avoid phagocytosis [47]. Pathogenicity of *V. tapetis*, mainly studied in Manila clam hemocytes, results in a

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

Conclusion

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

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