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Immune responses of phenoloxidase and superoxide dismutase in the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis* – Part II: Combined effect of temperature and two *V. tapetis* strains

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ABSTRACT

Manila clams, *Venerupis philippinarum* (Adams and Reeve, 1850), were experimentally infected with two different bacterial strains and challenged with two different temperatures. Bacterial strains used in this study were *Vibrio tapetis* strain CECT4600^T, the causative agent of Brown Ring Disease (BRD) and *V. tapetis* strain LP2, supposed less virulent to *V. philippinarum*. *V. tapetis* is considered to proliferate at low temperatures, i.e. under 21 °C. In a global warming context we could hypothesize a decrease of mass mortalities caused by *V. tapetis* but these thermal changes could also directly impact the immune system of the host *V. philippinarum*. Thus, the aim of this study was to investigate the effects of the extrapallial injection with *V. tapetis* combined with temperature challenge on two enzymes activities in *V. philippinarum*. More precisely, after infection, phenoloxidase (PO) and superoxide dismutase (SOD), two major enzymes involved in immune response, were studied for 30 days in two compartments: the mantle and the hemolymph. Conchyolin Deposit Stages (CDS) and Shell Repair Stages (SRS) were also determined 30 days post-injection as a proxy of the virulence of the tested strains. In this study, we highlighted that host–pathogen interaction in a varying environment affects the enzymatic response of the host. The coupled effect of *V. tapetis* injection and temperature challenge was detected 30 days post injection and resulted in virulence differences. These findings were supported by CDS and SRS determination in clams and lead to the conclusion that clam's immunity could be enhanced at 22 °C while *V. tapetis* virulence is lowered at this temperature. Another result of our study was the increase of PO and SOD basal activities as clams are exposed to warmer temperature.

1. Introduction

Global change is a real threat to marine invertebrates because increased atmospheric CO_2 is causing ocean warming and acidification [2,3]. In this climate change context, recent studies underlined the fact that mass mortality events and diseases are becoming more frequent in marine ecosystems [4–6]. Filter feeding invertebrates are particularly exposed to these diseases because to capture their food they have to filter great volumes of water, potentially containing microbes and bacteria, through their gills [7]. An example of marine invertebrate susceptibility to bacteria present in their environment, is the case of the Brown Ring Disease (BRD) affecting the manila clams *Venerupis philippinarum* (Adams & Reeve, 1850) along European coasts [8]. *V. philippinarum*, native to the Indo-Pacific was introduced in France between 1972 and 1975 for aquaculture purposes because of its faster growth rate compared to the indigenous clam, *Venerupis decussata* (Linnaeus, 1758) [8,9]. Fifteen years after its introduction in France, the first mortality events occurred in the first production site in Landeda (North Finistère, Brittany) [10,11]. These mortalities were associated with a characteristic symptom: an accumulation of a brown organic matrix on the peripheral inner shells [12] giving this disease its name: Brown Ring Disease (BRD). Since 1987, BRD spread along French and European coasts [8] and more recently, the disease symptoms have also been reported in clams in South Korea in 2003 [13] and in Japan in 2008 [14]. BRD is caused by the gram-negative bacterium *Vibrio tapetis* [11,15] which binds to the periostracal lamina of the manila clam and inhibits the normal process

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of biomineralization. This inhibition of shell synthesis leads to the formation of a brown organic deposit of conchiolin on the mantle edge and also a degradation of the periostracal lamina [16,17]. More precisely, conchiolin deposits form the first step of diseased animal defense enrobing bacteria [17]. A second step, in infected animals, results in the formation of new calcified layers above the conchiolin deposits. If the clam defense mechanisms are not sufficient, the development of the disease results in the penetration of *V. tapetis* in the extrapallial fluids and then, if tissue lesions occur, penetration into the tissues and into the hemolymph leading to animal death by a general infection [19,20].

This host–pathogen interaction is actually a tripartite interaction because the environmental temperature appears to be a strong influence factor on the progression of BRD as it impacts both on the pathogen and on the host. In fact, *V. tapetis* CECT4600^T (strain isolated in 1990 in France) has an optimal growth temperature of around 20 °C and temperatures greater than 27 °C are lethal for it [21,22]. The optimal temperature for the development of BRD is around 14 °C and between 15 and 21 °C the disease progression is negatively correlated with the temperature [21], which is why BRD is considered as a cold water disease and also why BRD events do not occur in tropical latitudes. The thermal factor also explains the annual cycle of disease observed in clam stocks: the number of diseased organisms increases during winter when water temperature is relatively low [1,23]. The environmental temperature also influences the host immune parameters since in clams maintained at 21 °C, infected with *V. tapetis* CECT4600^T, the dead hemocyte percentage is lower and Leucine AminoPeptidase (LAP) and lysozyme activities are higher than those of animals maintained at 14 or 18 °C [1]. Better shell repair at 21 °C confirms the hypothesis that temperature has a positive influence on the manila clam immune system [1].

Therefore, to better understand the impact of environmental factors, such as temperature, on disease it is not sufficient to study the response of each interaction player in isolation but is necessary to study both at the same time in synergy [24].

More specifically, the aim is to study the impact of water temperature on the immune system of *V. philippinarum* (here evaluated using two key enzyme activities) exposed to two pathogenic strains of *V. tapetis*, previously described as presenting different virulences [36,38]. Pathogenicity is defined as the microbe's capacity to induce disease or provoke damage in the host; this damage can result directly from pathogen's action or from immune response of host [25]. In our study, pathogenicity of *V.tapetis* strains is examined as the damages or symptoms of BRD developed in clams through the apparition of conchiolin deposits on the shell. Virulence involves both bacterium and host and is defined as the degree of pathogenicity or the relative capacity of a microbe to cause damage in the host [25,26]. Thus, virulence can be assessed by microbes' characteristics (such as virulence factors) or by host response analyze [25]. In the present work, the two *V. tapetis* strains' virulence was assessed through host response and more precisely through the degree of disease development in clams (Conchiolin Deposit Stages or CDS) and the degree of shell reparation (Shell Repair Stages or SRS) [8,18]. This virulence assessment is appropriate as *V. tapetis* proliferation in host is proportional to the symptoms' development degree [27].

To evaluate the immune capacity of the host species we decided to monitor two enzyme activities: phenoloxidase (PO) and superoxide dismutase (SOD). PO are copper-binding enzymes converting phenol compounds to unstable quinones. More precisely, phenoloxidases are divided in tyrosinases (E.C.1.14.18.1), which oxidize monophenols into o-diphenols; catecholases (E.C.1.10.3.1), which oxidize o-diphenols into o-quinones; and laccases (E.C. 1.10.3.2), which oxidize o-diphenols into o-quinones and p-diphenols into p-quinones. The produced quinones are then non-enzymatically polymerized into melanin and its derivatives [28–30]. These compounds are important elements of immune system with their fungistatic, bacteriostatic and antiviral properties [31] and POs are also key enzymes of invertebrate immune systems thanks to their involvement in self/non-self recognition, phagocytosis and nodule formation [32–34]. SOD (E.C. 1.15.1.1) is another oxidoreductase enzyme of immune system preventing reactive oxygen species (ROS) accumulation [35]. Both enzymatic activities have been followed through disease development and recovery in different parts of the animal: the mantle, which is the first tissue in contact with the bacterium after it binds to the periostracal lamina and the hemolymph which is the circulating fluid.

2. Materials and methods

2.1. Biological material and acclimation procedure

For this study, more than 1000 adult clams, *V. philippinarum*, were supplied by SATMAR (Aquaculture Company – Ile Tudy – South Finistère – France). These clams, 39.44 ± 1.96 mm length, were transferred to the laboratory (LEMAR - Institut Universitaire Européen de la Mer, Brest, France), divided into 18 aerated tanks and acclimated for one week before the injections. Nine of these tanks contained 15.3 ± 0.6 °C water and the 9 remaining tanks contained 22.0 ± 1.0 °C. For each tested water temperature, three conditions were applied and tested in triplicate: *V. tapetis* CECT4600^T injections, *V. tapetis* LP2 injections and Sterile Sea Water (SSW) injections (control).

2.2. Bacterial strains

For this study, the following cultured bacterial strains were used:

- *V. tapetis* CECT4600^T (junior synonym: *Vibrio* Prédominant 1: VP1), pathogenic agent of BRD in clams and isolated from diseased *V. philippinarum* in 1990 in Brittany (France; [15,17]). To date this strain is still considered as the most virulent *V. tapetis* strain to *V. philippinarum* [36].
- *V. tapetis* LP2, isolated from *Symphodus melops* in 1999, in Norway [37]. This strain is less virulent to *V. philippinarum* than the CECT4600^T strain after *in vivo* pallial cavity inoculation or after *in vitro* biotests [36,38].

These strains were cultured in Zobell's medium overnight at 18 °C and the bacterial-cell concentrations were determined by spectrophotometry at 490 nm (a correlation between direct counts of colony forming unit and optical density had been established previously).

2.3. Experimental injection

Animals were removed from their tank 12 h before injections and maintained at air temperature close to their respective tank temperature (i.e. 15 or 22 °C). Clams were replaced in sea water (at the water temperature according to their tank), just before injections in order to facilitate their opening. For each thermal condition, one third of the animals were injected with 100 μL of Sterile Sea Water (SSW), a third of animals were injected with 100 μL of *V. tapetis* CECT4600^T suspension (10^6 CFU mL^{-1}) and the last third of animals received 100 μL of *V. tapetis* LP2 suspension (10^6 CFU mL^{-1}). Injections were carried out, for the first time, in the peripheral extrapallial fluids localized under the mantle (between the pallial line and the edge of the shell) to allow *V. tapetis* strains to bypass the first biological barrier (mucus and periostracal lamina).