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External pH modulation during the growth of *Vibrio tapetis* , the etiological agent of Brown Ring Disease

Rahmani Alexandra [1, *], Mathien Clémentine [1], Bidault Adeline [2], Le Goïc Nelly [1], Paillard Christine [1, *], Pichereau Vianney [2, *]

[1] Univ Brest ,CNRS IRD, Ifremer UMR 6539 LEMAR F-29280 Plouzané, France
[2] Univ Brest ,CNRS IRD, Ifremer UMR 6539 LEMAR F-29280 Plouzané, France

* Corresponding authors : addresses mail : alexandra.rahmani@univ-brest.fr ; christine.paillard@univ-brest.fr ; vianney.pichereau@univ-brest.fr

Abstract :

Aims

Brown Ring Disease (BRD) is an infection of the Manila clam *Ruditapes philippinarum* due to the pathogen *Vibrio tapetis*. During BRD, clams are facing immunodepression and shell biomineralization alteration. In this paper, we studied the role of pH on the growth of the pathogen and formulated hypothesis on the establishment of BRD by *V. tapetis*.

Methods and Results

In this study, we monitored the evolution of pH during the growth of *V. tapetis* in a range of pH and temperatures. We also measured the pH of Manila clam hemolymph and extrapallial fluids during infection by *V. tapetis*. We highlighted that V. tapetis modulates the external pH during its growth, to a value of 7.70. During the development of BRD, *V. tapetis* also influences extrapallial fluids and hemolymph pH in vitro in the first hours of exposure and *in vivo* after 3 days of infection.

Conclusions

Our experiments have shown a close interaction between *V. tapetis* CECT4600, a pathogen of Manila clam that induces BRD, and the pH of different compartments of the animals during infection. These results indicate that that the bacterium, through a direct mechanism or as a consequence of physiological changes encountered in the animal during infection, is able to interfere with the pH of Manila clam fluids. This pH modification might promote the infection process or at least create an imbalance within the animal, that would favor its persistence. This last hypothesis should be tested in future experiment.

Significance and Impact of Study

This study is the first observation of pH modifications in the context of BRD and might orient future research on the fine mechanisms of pH modulation associated to BRD.

Keywords : BRD, V. tapetis, R. philippinarum, pH, modulation effect, pH modifications, neutralizing activity

INTRODUCTION

The Manila clam *Ruditapes philippinarum* is a bivalve mollusc species of significant economic interest with a world production mainly from China, followed by European countries such as Italy, Spain and France (Paillard 2004b). It was imported in France in the early 70's for aquaculture. Ten years later, mortalities have been observed in clam's cultures, caused by an

infection called the Brown Ring Disease or BRD (Paillard et al. 1989). Indeed, infected clams were characterized by a brown deposit in the inner face of the shell between the pallial line and the edge of the shell (Paillard et al. 1994). BRD is caused by a Gram negative bacterium, *Vibrio tapetis* (Paillard and Maes 1990; Borrego et al. 1996). BRD has caused significant economic losses in livestock farms on the Northern European coasts because the pathogen has an optimum infection rate in cold waters at around 14°C. For example, in France, Manila clam production decreased from 500 tons in 1987 to 200 tons in 1989, the aquaculture of clams production being mainly located in Brittany (France), an area highly sensitive to BRD (Paillard 2004b).

V. tapetis is a microparasite acting in two modes. In the case of BRD, the bacterium is referred to as an external microparasite because it degrades the periostracal lamina of the clam to enter the extrapallial compartment without invading the tissues. In this case, the pathogen induces BRD in Manila clams. In some cases, when tissues are damaged, *V. tapetis* acts as an internal microparasite by infiltrating the animal's tissues, resulting in mortality within days (Paillard 2004a).

During infection by *V. tapetis*, the pathogen therefore colonizes the periostracum and degrades it in order to infiltrate peripheral or even central extrapallial fluids (EPFs). As a result, it inhibits the biomineralization of the shell, preventing the formation of calcium carbonate crystals of aragonite and thus leads to the accumulation of the organic brown deposit (Paillard et al. 1994). Furthermore, infected clams are facing an immunodepression caused by *V. tapetis*. Hemocytes, which are key effectors of the immune defenses of clams, are altered in BRD by actin cytoskeleton disorganization causing rounding of hemocytes and downregulation of pathogen recognition effectors. Consequently, infected hemocytes are no more able to ensure phagocytic progress to degrade the pathogen (Choquet et al. 2003; Allam et al. 2014; Rahmani et al. 2019).

Many factors are involved in BRD. Indeed, some alterations on the inner shell have significant impacts on the functioning of the affected clams. Impeding the functioning of adductor muscles and siphons, they result in abnormal positioning of the clam in the sediment and prevent the proper closure of valves that can lead to mortality when fishing or directly in the sediment, as clams can no longer retain their inter-valve water (Goulletquer et al. 1989; Paillard 2016).

Shell biomineralization is characterized by the formation of Calcium-Carbonate crystals ($CaCO_3$) from calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions. $CaCO_3$ crystals are the main constituent of shellfish. In the Manila clam, the shell is composed of aragonitic crystals (Taylor, J D et al. 1973; Trinkler et al. 2011). The saturation state of this mineral in seawater determines whether the biomineralization reaction is favored in the direction of crystal formation or dissolution. It is defined by the concentrations of Ca^{2+} and CO_3^{2-} ions.

Biomineralization process is often studied in the context of ocean acidification because pH is a factor that considerably influences the physiology of marine calcified organisms, and especially this crystals formation. Indeed, acidification has the effect of reducing the saturation rate of calcium carbonate crystals through the reduction in available CO_3^{2-} ions, thus promoting the dissolution of the shell (Gattuso and Hansson 2011). In the mussel *Mytilus edulis*, decrease in pH leads to a decrease in the net calcification rate, Ca^{2+} ion content and, in general, in a change in the ultrastructure of the shell and a decrease in amino acid content (Li et al. 2015). Combination of temperature and pCO_2 increase results in a decreased shell hardness in the clam *Mercenaria mercenaria* and in the American oyster *Crassostrea virginica*, indicating major changes in the biomineralization process (Ivanina et al. 2013). In the brittle stars *Amphiura filiformis*, authors showed that the animal was able to compensate, in the short term, for the effects of pH decline by accelerating calcification and metabolism at the expense of certain functions such as growth and reproduction because of the high energy cost required for this mechanism (Wood et al. 2008). In summary, most of the studies reflect that ocean acidification reduces the calcification rates of animals composed of calcitic or aragonitic crystal shells by promoting the dissolution reaction of these crystals.

In the Manila clams, calcification, i.e. shell biomineralization reaction, occurs in extrapallial fluids: ie the mantle secretes the shell components, the organic matrix (conchiolin) and the calcium carbonate that form the aragonitic crystal shell (Paillard and Le Pennec 1993; Trinkler 2009; Trinkler et al. 2011). The periostracum (outer part of the shell) is made of a sclerotinized organic matrix. Shell organic matrix is mainly composed under normal conditions of aspartic acid, glycine and serine, responsible for binding with calcium ions: chelation by sulphate ester groups on polysaccharide chains would initiate biomineralization. When BRD develops, affected

clams have deficiencies in amino acid such as glutamic acid, aspartic acid, serine, and alanine, which can lead to poor binding to Calcium - Carbonate (Goulletquer et al. 1989).

Given the importance of pH homeostasis in the biomineralization process, it seems likely that pH might play a significant role in the pathogenicity of *V. tapetis* in the case of BRD. Nevertheless, the influence of pH on the development of *V. tapetis*, as well as on the development of BRD, have never been investigated. In this paper, we chose to investigate the influence of pH on the development of the pathogen and the modulation of pH associated to this bacterium in clams' fluids. Furthermore, we monitored for the first time the pH modification in clam fluids during *in vivo* challenge by *V. tapetis* allowing us to formulate hypothesis that pH variations due to *V. tapetis* might be a virulence strategy of this pathogen linked to pathogenicity and to its ability to alters biomineralization. This study is the first investigation of pH modifications in the context of BRD.

Materials and methods

1) Monitoring of the pH modifications during growth of *V. tapetis* in culture media

Culture media and bacterial strain: The bacterial strain used in these experiments is *V. tapetis* CECT4600, grown in Zobell medium (Zobell 1941) and incubated at 18°C. The first objective was to monitor the growth of this strain at 7 different pH values and 4 different temperatures. The 7 liquid culture media used in this experiment were obtained from Zobell media supplemented with NaOH 1 M or HCl 0.1 M until the expected pH is obtained. After autoclave sterilization, the pHs of the 7 media were 6.00, 6.45, 7.00, 7.55 (not modified, control medium, CM), 7.86, 8.12 and 8.70, respectively. pH of the cultures/media were measured by using a Mettler Toledo© InLab Micro electrode after calibration of the electrode with pH buffers 4, 7 and 9 at the beginning and the end of each experiment. The 4 temperatures tested were 14°C (optimal infection temperature), 18°C (optimal growth temperature), 21°C (temperature at which the recovery process occurs mainly) and finally 27°C (temperature at which the bacteria die) (Borrego et al. 1996; Paillard et al. 2004; Trinkler 2009; Paillard 2016).

Growth experiments: colonies of strain CECT4600 were resuspended in 2 ml of Zobell medium (unmodified pH, control medium CM). The Optical Density (OD) was measured at a wavelength of 492 nm and the suspension concentration was adjusted to an OD of about 0.1, i.e. to a cell density of 9.4×10^7 CFU.ml^{-1}. The suspension was diluted at 1:100 in 3 ml of Zobell medium at the desired pH. The control samples were the same sterile media. 400 µL of inoculated media or controls were distributed in a Bioscreen© 100-wells plate (5 replicates per tested condition), allowing a sterile, thermally controlled culture with an OD measurement at regular intervals under stirring. This protocol was repeated twice; each experiment was run for 24 hours (except at 14°C where the experiment ran for 30 h to take into account the reduced bacterial growth), in a shaking mode, with measurement of OD every 20 minutes at a wavelength of 492 nm ; temperature was set up at either 14, 18, 21 or 27°C. To represents the pH measurement at the beginning of the growth experiment (T0) and TF (TFinal) represent the pH measurement at the end of experiment, depending on the different conditions as detailed above.

pH monitoring during the bacterial growth: Two precultures of *V. tapetis* CECT4600 were performed in Zobell broth during 15 h at 18°C and 24 h at 14°C in order to reach an OD of 0.98. Each preculture was diluted 12 times (initial OD = 0.08) with fresh Zobell medium at the different pHs (from 6.00 to 8.70). With these new cultures, bacterial growth was performed under shaking at 14°and 18°C, respectively from the corresponding pre-culture, in three replicates. The pH was measured, from 200 µl-samples, at the beginning of the experiment, every hour during the exponential growth phase, and finally in the stationary phase (i.e. 48 h of growth at 14°C and 24 h of growth at 18°C).

2) Monitoring of the pH in extrapallial fluids (EPFs) and hemolymph of Manila clams during infection by *V. tapetis*

Animals: Animals used in this study were adult Manila clams (about 40 mm) from Landeda (Finistère, France), kindly provided by the company SATMAR. One pool of 40 clams (for the *in vitro* study of fluids) and 2 pools of 100 clams (for the *in vivo* infection study) were acclimatized for 3 and 2 weeks, respectively, in oxygenated seawater at 14°C.

Fluids and shell sampling: The fluids chosen for this experiment were hemolymph and EPFs. Hemolymph, EPFs and shell were randomly collected extemporaneously from 10 clams. Hemolymphs have been collected from the adductor muscle and EPFs from the extrapallial cavity of the animals as previously described (Le Bris et al. 2015). The quality of the hemolymph samples was checked under the microscope (presence of pseudopods, unrounded hemocytes, serum and hemocytes without microorganism contaminations) then the samples were pooled and the number of hemocytes was determined by using a Malassez counting grid. Shell parts were broken with a hammer to obtain small pieces and were sterilized by UV (wavelength 254 nm) for 15 minutes. EPFs were also pooled. The hemocytes concentration in the hemolymph pool was 5.45×10^5 hemocyte per ml.

For *in vitro* experiments, 150 µl of FSSW, hemolymph or EPFs were placed in 1.5 ml sterile tubes, with supplementation of powdered Manila clam shell, in the same amount in each sample, in

188 order to design an *in vitro* experiment that is as close as possible of natural conditions and to
189 allow ion exchanges between fluids and shell that might be dependent of the *V. tapetis* growth.
190 Then, 200 µL of a *V. tapetis* CECT4600 suspension in FSSW was added to each tube, in order to
191 reach a final bacteria:hemocytes ratio of 25:1 (1.37×10^7 CFU.ml^{-1}); 200 µl of FSSW was added for
192 the controls. Each condition was tested in triplicates. pH measures were performed at times 0 h,
193 3 h, 24 h, 48 h and 72 h and tubes were shaken before measurement.
194
195 For *in vivo* infection experiments, the protocol was followed as previously described (Paillard
196 and Maes 1994). The animals were first left out of the water during the night before infection to
197 allow the valves to open spontaneously in water, and then injected with 100 µl of FSSW for 50
198 control individuals, or 100 µl of bacterial suspension (5×10^8 CFU.ml^{-1} in FSSW) for 50 infected
199 individuals, in the pallial cavity. The injected animals were left out of water for 3 additional hours
200 to allow the pathogen to colonize them, and then were transferred again into their respective
201 seawater tanks. Animals were sampled at different time points after infection, ie. after 15 hours,
202 1 day, 3 days, 7 days, 14 days and 21 days post injection (d.p.i.). pH of the EPFs and hemolymph
203 samples were measured by using the Mettler Toledo microelectrode calibrated with pH 4, 7 and
204 9 buffers. The initial sample (T0) was taken just before injection. The mode of infection does not
205 induce mortality and allows to reproduce first stage of BRD to see the effect of *V. tapetis,* even
206 before the development of organic deposit in the shell, highlighting the pH variations that
207 occurs in the really first days of *V. tapetis* infection.
208
209 *Statistical analyses:* Statistical analyses were performed by using a Student test or a Mann
210 Whitney Wilcoxon test, depending of data normality.

RESULTS

V. tapetis CECT4600 modulates the pH of Zobell culture medium during its growth.

V. tapetis was cultured in Zobell broths which pH were adjusted from 6.00 to 8.70, at 4 different temperatures ranging from 14°C to 27°C. The results obtained are reported in Table 1. First, we observed that the bacterium is able to grow at temperatures ranging from 14°C to 21°C, in the pH range tested, but not at 27°C. In addition, consistently with the results previously published by Borrego et al (1996), the optimal growth temperature is 18°C in the unmodified medium.

V. tapetis has a high tolerance to pH because we observed a growth in all the tested culture media. However, at 14°C, the fastest growth was obtained for the control medium (pH 7.4, DT = 3.15 h), but growths were observed over a pH range from 6.45 to 8.12, with doubling times from 3.15 to 3.5 and max ODs higher than 1, while the growth rates were lower at the extreme pHs tested, ie. 6 and 8.7 (Table 1). The best growth at 14°C was observed in the unmodified culture medium (MC, with a DT of 3.15h), and at pH 7.86 at 18°C (DT = 1.79h). Interestingly, at the growth temperatures 18 and 21°C, very good growth parameters were obtained for more acidic pHs, from which the lowest DTs were observed for the pH value 6.45 (DTs = 1.85 and 1.93 h at 18 and 21°C, respectively).

The pHs of the culture media have been measured at both the beginning (To) and the end (TF) of bacterial growth in all the conditions tested. Results are presented in Figures 1 to 3. In the control samples (no bacteria), the pHs did not change significantly between the measurements made at To and TF (Fig. 1 to 3). By contrast, after growth of *V. tapetis* at 14, 18 and 21°C, the pH changed during growth to reach an average value of 7.90 in all media tested (Fig. 1 and 2). The pH modifications are the same at these three temperatures, allowing us to show them all in the same figures (Fig. 1 and 2). We did not observe such a pH modification of the culture medium at 27°C, a condition where no growth of the bacterium was observed (Fig. 3).

Figure 2 shows the statistical distribution of pH values measured at To and TF in all media. The mean and variance of this distribution differed significantly during the experiment only when the bacterium was able to grow (*pvalue* < 0.0001). Overall, these results show that *V. tapetis* is able to modify the pH of its culture medium during its growth towards a pH close to 7.90.

We measured the evolution of medium pH all along the exponential growth phase of *V. tapetis* at 14°C (Fig. 4) and 18°C (Fig. 5). In the control samples, the pH changed only slightly during the experiment, resulting in very low slope curves (data not shown). By contrast, in the experimental samples, during the growth of *V. tapetis*, the pHs of the media (which were initially 6 to 8.7) converged to an average value of 7.71, whatever the temperature of growth (Fig. 4 and 5). Therefore, our experiments show that *V. tapetis* is able, during its growth, to modify the pH of its culture medium. More precisely, *V. tapetis* tends to 'neutralize' culture media during its growth, by acidifying alkaline media and alkalinizing acidic media, to make it reach a mean value of 7.7-7.9 whatever the initial pH of the culture medium. However, this effect can be due to either a passive mechanism as a consequence of the bacterium's growth or an active mechanism that allow the pathogen to infect the Manila clam.

External pH modulation during the in vitro growth of V. tapetis CECT4600 in clams extrapallial fluids or hemolymph.

We aimed at determining whether the bacterium can also modulate the pH of clam biological fluids in which *V. tapetis* is known to proliferate during the infection, ie. the EPFs and the hemolymph. In these experiments, FSSW was used as the reference fluid because it does not allow any growth of *V. tapetis*. The same amount of powdered shell fragments was added to each sample in order to design an *in vitro* experiment that is as close as possible of natural conditions and to allow ions exchanges between fluids and shell that might influence *V. tapetis* growth. The obtained results are shown in the Figure 6. Our experiments showed that in FSSW, the presence of *V. tapetis* did not lead to any significant change in pH as compare to the control, at each time point tested. By contrast, in EPFs and hemolymph, exposure to *V. tapetis* led to a significant decrease of pH as compared to the initial conditions after 3 hours of exposure (*pvalue* < *0.001*). Indeed, for both clam biological fluids (ie. EPFs and hemolymph), the pH significantly dropped from 7.6 to 7.4 after three hours of *Vibrio* exposure, and then stabilized at approx. 7.25. However, for longer times, the pH also tended to decrease in the controls, so as no significant difference appears between the control samples and the samples exposed to *V. tapetis* for the last time points, thus revealing an interaction only in the first hours of exposure.

Extrapallial fluids and hemolymph pH modulation during in vivo challenge of the Manila clams by V. tapetis CECT4600.

The previous demonstration that the presence of *V. tapetis* can interfere *in vitro* with the pH of these clam biological fluids led us to hypothesize that this could happen *in vivo*, and that the modulation of pH of these fluids could be an important aspect of the infection process. Monitoring of pH evolution in EPFs and hemolymph after Manila clams infection by *V. tapetis* is presented in Figures 7 and 8, respectively. Both fluids displayed a relatively similar pH evolution following the injection of FSSW or *V. tapetis* in the animals. Considering the whole *in vivo* infection experiment (22 days), we did not observe any significant difference in pH between the control and infected animals for both fluids. However, we observed a weak but significant difference between the pHs of the EPFs and hemolymph from control and infected clams at 3 days post injection (dpi). Indeed, the pH of these fluids were higher (of approx. 0.2 pH unit) in both cases in clams injected with a suspension of *V. tapetis*, as compared to clams injected with FSSW ($pvalue < 0.02$). Our results then tend to suggest that exposure to *V. tapetis* induce physiological changes in challenged Manila clam that influence the pH of EPF and hemolymph at 3 days of exposure to the pathogen. This also suggests that this particular period should be more precisely investigated during the study of host-pathogen interaction.

DISCUSSION

pH homeostasis is an essential aspect of cell physiology. It is also one of the main environmental factors controlling the biomineralization of calcified organisms. This explains, at least in part, the current abundance of studies dealing with the impact of ocean acidification on marine mollusks and other calcified marine organisms (Gattuso and Hansson 2011). The relation between decrease of pH and an increase of temperature has been studied in several bivalves species, showing that these two factors can affect the immune response as in the Mediterranean mussel *Mytilus galloprovincialis* (Matozzo et al. 2012) and was also shown to influence the abundance of parasites and incidence of bacteria as in the blue mussel *Mytilus edulis* (Mackenzie et al. 2014). Finally, pH adaptation is a crucial component of virulence of many pathogenic bacteria. In addition to having to survive the acid digestive barriers in some hosts, they must be able to tolerate acid stress resulting from lysosomal activity after being phagocytized by immune system cells such as macrophages or hemocytes (Asplund et al. 2014).

EPFs and hemolymph are involved in shell biomineralization and in immunity of the Manila clam, respectively, and have therefore a crucial importance in the development of BRD. BRD is typically a disease of clam biomineralization (Paillard et al. 1994). It is also a bacterial disease, in which the phenomenon of phagocytosis, and more specifically the phagosome-lysosome fusion, has a particular importance in the animal's immunity (Paillard 2004; Rahmani et al., 2019). For all these reasons, it is surprising that the effect of pH on *V. tapetis* has never been considered to date in the context of the BRD. In this study, we first aim to characterize the relationship between external medium pH and the growth of the pathogen *V. tapetis*.

We observed that *V. tapetis*, the bacterial etiological agent of BRD, is able to grow over a wide range of pHs, including at a cold temperature (14°C) which is an optimum for BRD development (Paillard et al. 2004). Interestingly, we showed that the bacterium, during its growth, modifies the pH of its environment, increasing it in acidic media, or acidifying it in alkaline ones, and systematically replaced the external pH to a value close to 7.7 during its growth in a kind of 'neutralizing activity' in the tested range. We further characterized this last point, by measuring *in vitro* the impact of the bacterial growth on the pH of the main biological fluids of the Manila clams, ie. extrapallial fluids and hemolymph, and then *in vivo*, the pH of these fluids in a context

315 of infection by inducing the main mode of infection of *V. tapetis*, allowing the pathogen to
316 colonize extrapallial fluids. We observed that at three hours after exposure to *V. tapetis,* the pH
317 of the two fluids decreased significantly more than that of the control in presence of powdered
318 Manila clam shell. EPFs and hemolymph contain hemocytes, which are the main cellular actors
319 of the immune system and one of the main targets of the pathogen.
320
321 In a previous transcriptomic analysis, we showed that *V. tapetis* is able to induce deregulations
322 of clam hemocytes physiology as reorganization of actin cytoskeleton, reduction of lysosomal
323 activity and down regulation of genes related to the complement pathway (Rahmani et al.
324 2019). In another previous study, our team had demonstrated that the cytotoxicity of the
325 bacterium to hemocytes was maximal, *in vitro*, after three hours of exposure to the pathogen
326 (Choquet et al. 2003). This phenomenon is reproduced by exposing Manila clam hemocytes to *V.*
327 *tapetis* during precisely three hours. The decrease in pH in the first three hours of exposure could
328 be due to the growth of the pathogen and could be related (either as a cause or a consequence)
329 to its cytotoxic effect on clam hemocytes where the influence of the bacterium is maximum.
330
331 The relationship between pH regulation and virulence is not always clear since the regulation of
332 the internal pH is a necessity for all cell types. Bacteria regulate their intracellular pH by using
333 proton pumps, or by transporting and/or metabolizing several acid or base compounds. For
334 example, *Escherichia coli* response to acid stress involves *amino acid decarboxylase antiporter*
335 *pairs* and *proton-pumping respiratory chains complexes* causing H+ ion efflux outside the cell. On
336 the contrary, during alkaline stress, *E. coli* was found to have a strong activation of *NhaA* Na^+/H^+
337 *antiporter*, an up-regulation of ATP synthetase leading to a strong entry of H+ ion into the cell
338 (Krulwich et al. 2011). However, intestinal bacteria are facing larger pH ranges than marine
339 ecosystems. NhaA antiporter is also present in the genome of *V. tapetis* CECT4600 according to
340 the recently published annotation (Dias et al. 2018), as well as calcium or potassium proton
341 antiporters. Furthermore, the presence of an enzyme such as arginine deiminase in the genome
342 of this pathogen might explain alkalinization of external pH observed during acidic stress as
343 already shown in another bacterium (Budin-Verneuil et al. 2006).

Regardless of this internal pH regulation, many bacteria modify the pH of their environment as a result of their energy metabolism. This is particularly the case for many fermentative bacteria producing large quantities of organic acids (e.g. lactic acid, propionic acid, formic acid, etc.) leading to an acidification of their environment (Nuryana et al. 2019). Other bacteria are known to alkalinize their environment, by using organic acids (e.g. lactate, aspartate, glutamate) as carbon and energy sources (Stancik et al. 2002). Nevertheless, these metabolisms are very dependent on the nature of the substrates used by the bacteria, and there are no case, to our knowledge, of bacteria capable of being both acidifying and alkalinizing in the same culture medium, only depending on the initial pH of that medium.

This discovery raises many questions about the *V. tapetis*'s adaptation mechanisms to pH stress, as well as about the energy metabolism(s) it uses, and the substrates it degrades from the Zobell medium, at the different pHs. This also raises important questions regarding the involvement of this 'neutralizing activity' in the expression of its pathogenicity, in the context of BRD development. The mechanisms related to this activity are for now unknown and might be due to several type of mechanisms as described above. Nevertheless, this study has revealed new, previously unsuspected problematics related to pH modulation, highlighting the need for further analysis of genes expressed during growth in order to elucidate the mechanisms linked to this phenomenon.

To better understand the close interaction between the clam and the pathogen in this context, we need to refer to BRD dynamics. BRD is a chronic infection, as the pathogen does not induce mortalities in the classical way of infection and can persist in infected animals (Paillard 2016). Indeed, the acute phase of the infection is divided into two parts: a first one characterized by an increase of *V. tapetis* concentration followed by the production of a brown deposit on the inner part of the shell, and a second one, which is not always present, characterized by shell repair that can lead to the complete remission of clams (Paillard 2016). In this study, we have performed injection of *V. tapetis* in the pallial cavity. This mode of infection allows recreating the first stages of BRD by allowing the pathogen to reproduce all the early steps of BRD and then induce an acute phase of infection until formation of the brown deposit. Dynamics of BRD and kinetics of *V. tapetis* in EPFs have previously been well characterized. In the first hours of

374 infection, the pathogen colonizes and degrades the periostracal lamina in order to enter
375 extrapallial fluids, and then colonizes the shell secretion (Paillard and Maes 1995), leading to an
376 increase of *V. tapetis* concentration in EPFs. Between 2 days and 7 d.p.i. the EPFs concentration
377 of *V. tapetis* reaches its maximum value (Bidault et al. 2015).
378 This particular period corresponds to an increase of pH in EPFs according to our study where
379 significant differences of pH have been observed in infected animals after 3 days (*in vivo*). It
380 should also be noted that an increase of pH has also been reported in hemolymph at 3 d.p.i.,
381 thus probably revealing the changes that occurs in this compartment at the biochemical level
382 during BRD (Allam et al. 2006).
383
384 In addition, we know that during infection, major changes occur in EPFs at the enzymatic level
385 with, for example, an increase in the activity of phenoloxidase, an enzyme involved in the
386 humoral response and melanization which leads to the brown deposit production (Söderhäll and
387 Cerenius 1998). Previous studies showed that phenoloxidase activity is sensitive to abiotic
388 factors such as pH (Le Bris 2013). Indeed, phenoloxidase activity decrease with pH in a range of
389 physiological values in the Atlantic blue crab *Callinectes sapidus*, mostly in the hemolymph
390 compartment (Tanner et al. 2006). Thus, increasing the pH in a range of physiological values in
391 both hemolymph and EFPs could modulate phenoloxidase activity in these two compartments.
392 As phenoloxidase is related to melanization process, the increase of pH can also modulate
393 melanization, associated to the bacterial embedding within shell matrix.
394
395 Considering these informations, the fact that *V. tapetis* is able to modulate the pH of such
396 compartments of the Manila clam in the context of BRD then questioned the impact of this
397 factor in the development of BRD itself. Indeed, pH is known to modulate the virulence of many
398 pathogenic bacteria. In *Vibrio cholerae*, for example, it has been shown that the protein ToxR,
399 which is responsible for the regulation of many virulence genes, is also strongly involved in the
400 response to acid stress, suggesting a close relationship between the virulence and the acid stress
401 response in this pathogen (Merrell and Camilli 2002; Lund et al. 2014). In *Pseudomonas*
402 *aeruginosa*, it has been shown that the infection was attenuated at pH 6 rather than pH 7.6, and
403 that the bacterium expresses genes that allow it to alkalinize the medium at acidic pH, while at

neutral pH, it preferentially expresses iron metabolism-related genes (Romanowski et al. 2011). In the case of *V. tapetis*, we don't know yet if pH variations are due to sensing of external pH by *V. tapetis* that induce a change in response, maybe in relation with its pathogenic activity or to the growth of the pathogen. This mechanism should be investigated in a future study.

To summarize, after investigation for pH modulation during interaction between the Manila clam and its pathogen *V. tapetis*, our results indicate that *V. tapetis* is able to modulate the pH by a "neutralizing activity" during its growth. For the first time, we highlighted this activity in both *in vitro* challenge and *in vivo* infection of Manila clam. We have determined that this interaction occurs at a very precise time (3 hours exposure *in vitro* and 3 days *in vivo*). These special time periods correspond to the main interactions between the pathogen and the clam hemocytes. It is then likely that these close interactions might play a role in the first steps of BRD development. Nevertheless, the mechanisms related to this phenomenon are, for now, unknown. Our study is the first evidence that pH modulations might be a novel, and since now undiscovered, mechanism that will help us to better understand host-pathogen interaction in the context of BRD. Moreover, it allows to focus on really precise time period by both *in vitro* and *in vivo* challenges in order to better characterize these particular interactions and to understand the mechanisms and the importance of pH modulation in the pathogenic activity of *V. tapetis*.

AUTHORS CONTRIBUTIONS

AR designed the protocols with help of AB, NLG and CP and VP. AR performed *in vitro* experiments on *V. tapetis*. AR and CM performed *in vitro* and *in vivo* experiment on Manila clams challenged by *V. tapetis*. AR, VP and CP wrote the article (the original draft was written by AR). This article was carefully reviewed by other co-authors, who all approved the final version.

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CONFLICT OF INTEREST

No conflict of interest declared.

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FIGURES AND TABLES

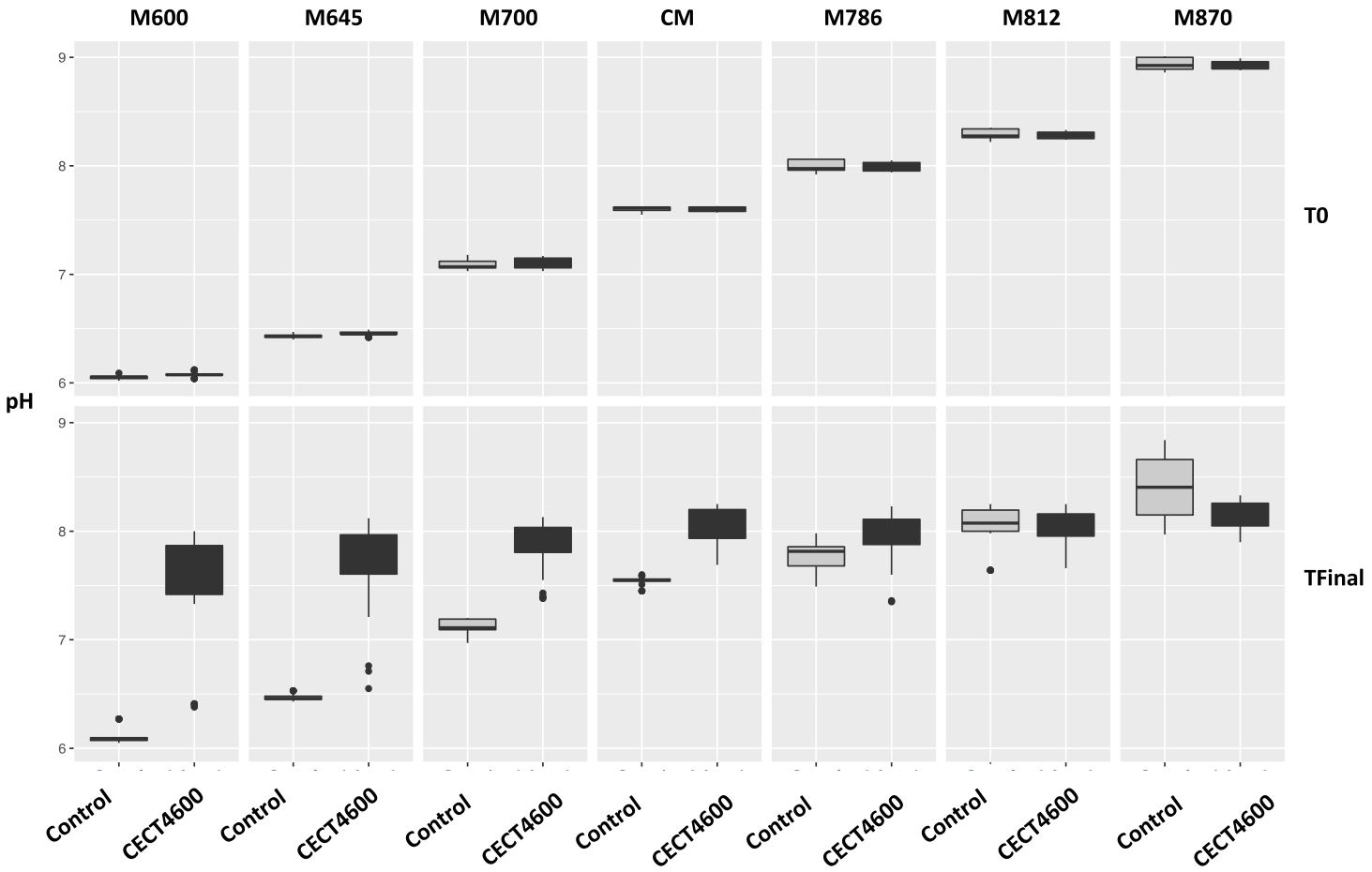

Figure 1: pH changes in a *V. tapetis* CECT4600 culture after growth at 14°C, 18° and 21°C in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) (15 replicates)

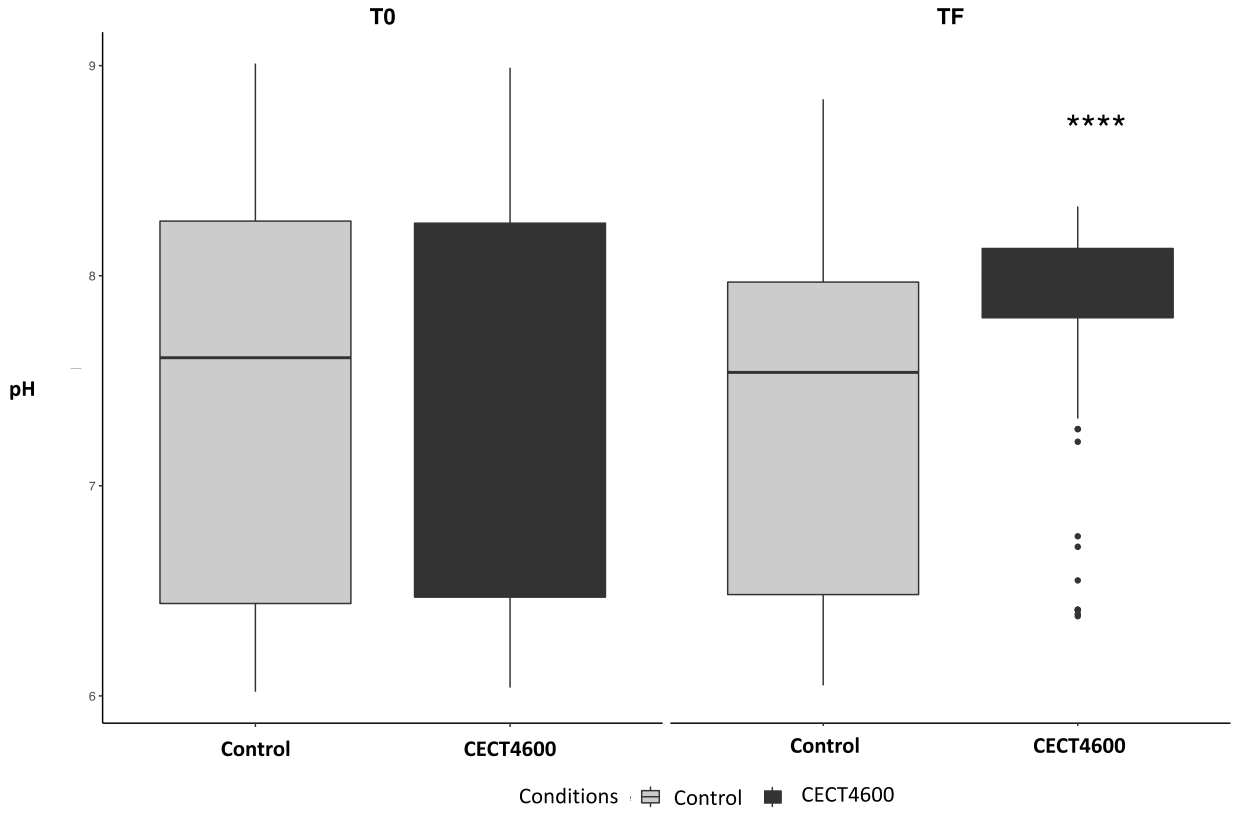

Figure 2: pH variance of a *V. tapetis* CECT4600 culture after growth at 14°, 18° and 21°C in pH adjusted Zobell media from 6.00 (M600) to 8.70 (M870) (15 replicates). Left : To ; Right : TF or TFinal

**** : pvalue < 0.0001, Student test

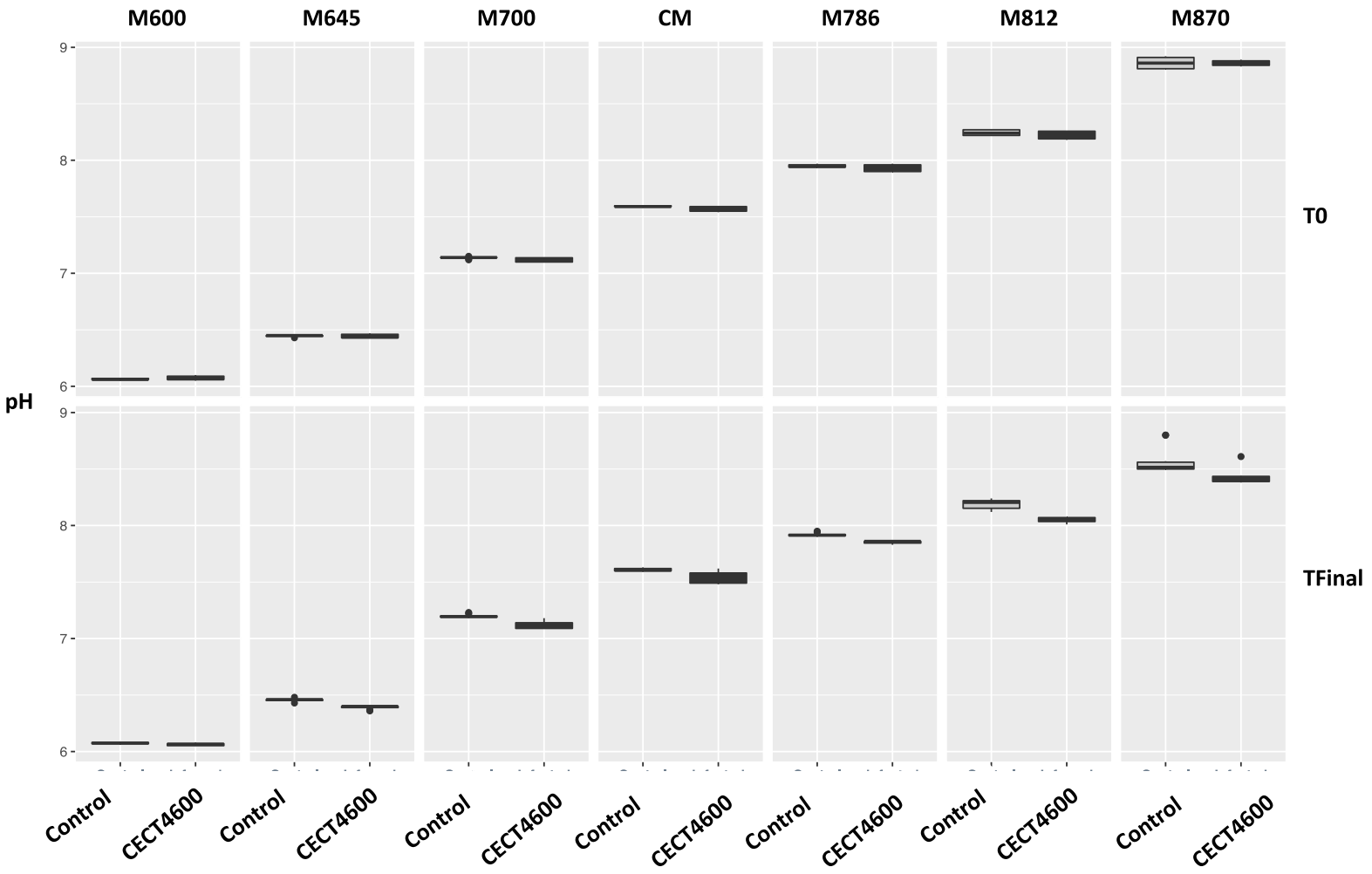

Figure 3: pH changes in a *V. tapetis* CECT4600 culture after growth at 27° in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) (15 replicates)

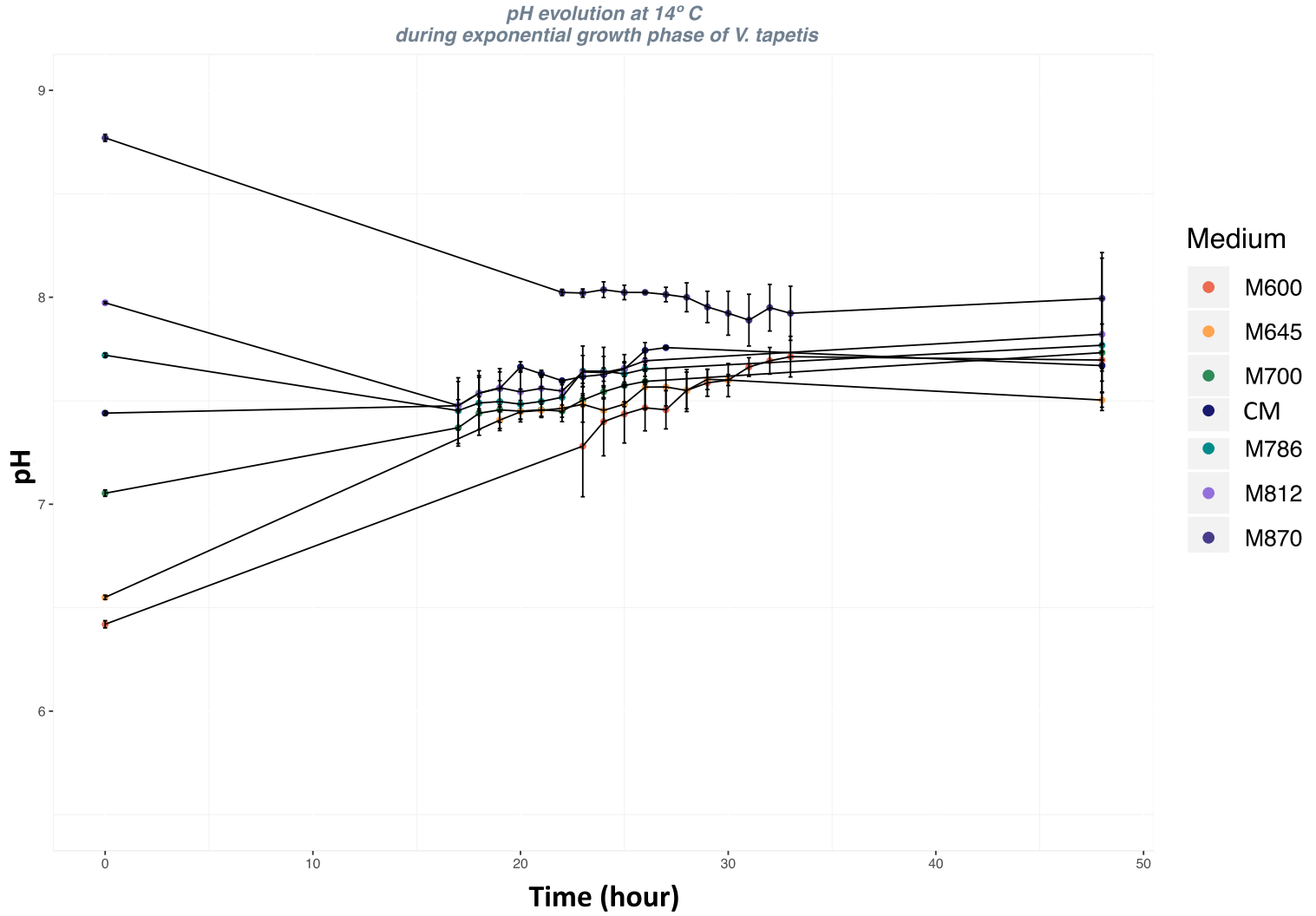

Figure 4: pH change in a culture of *V. tapetis* during the exponential growth phase at 14°C, in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) ; CM is the original medium where pH was not modified.

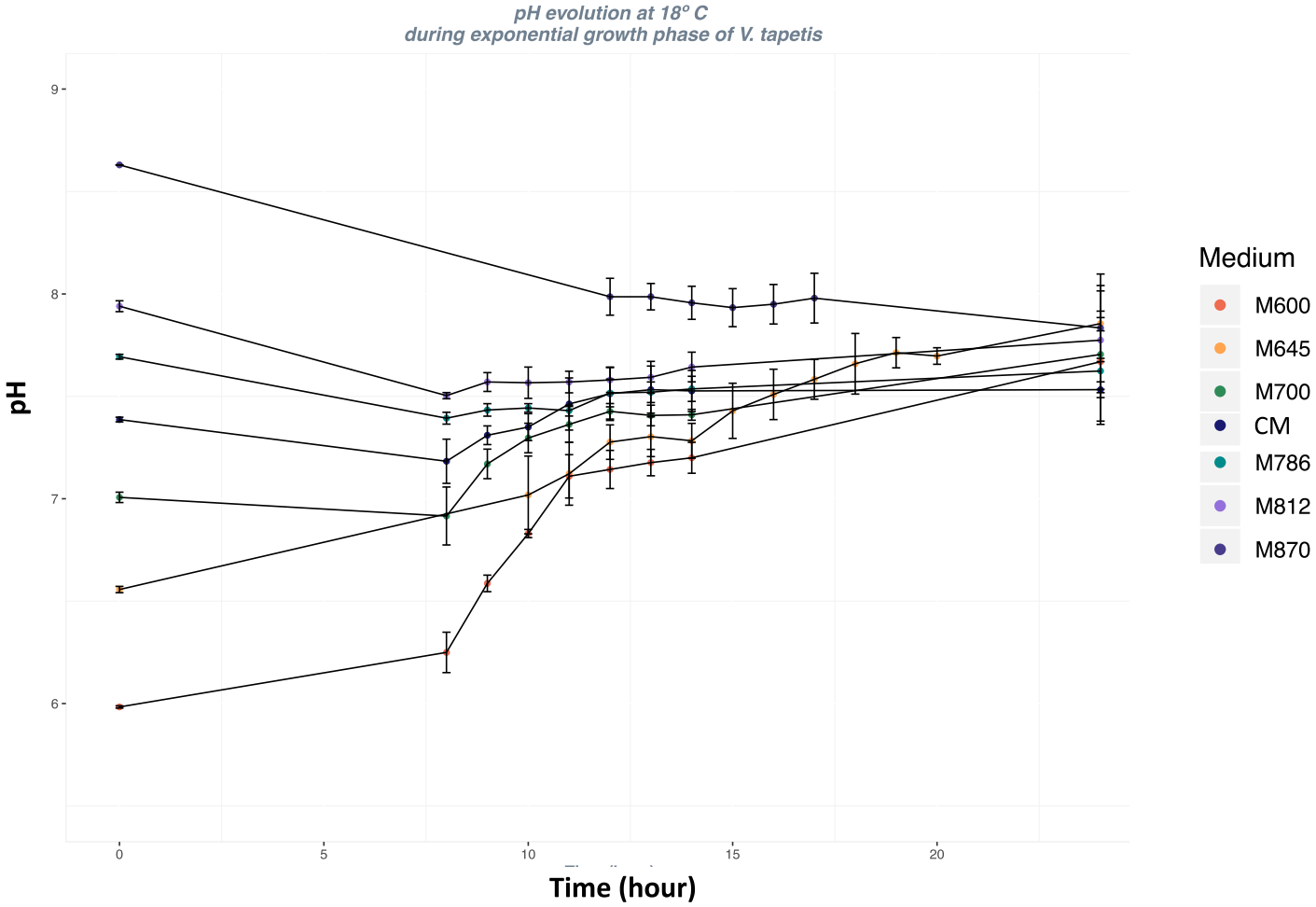

Figure 5: pH change in a culture of *V. tapetis* during the exponential growth phase at 18°C, Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) ; CM is the original medium where pH was not modified.

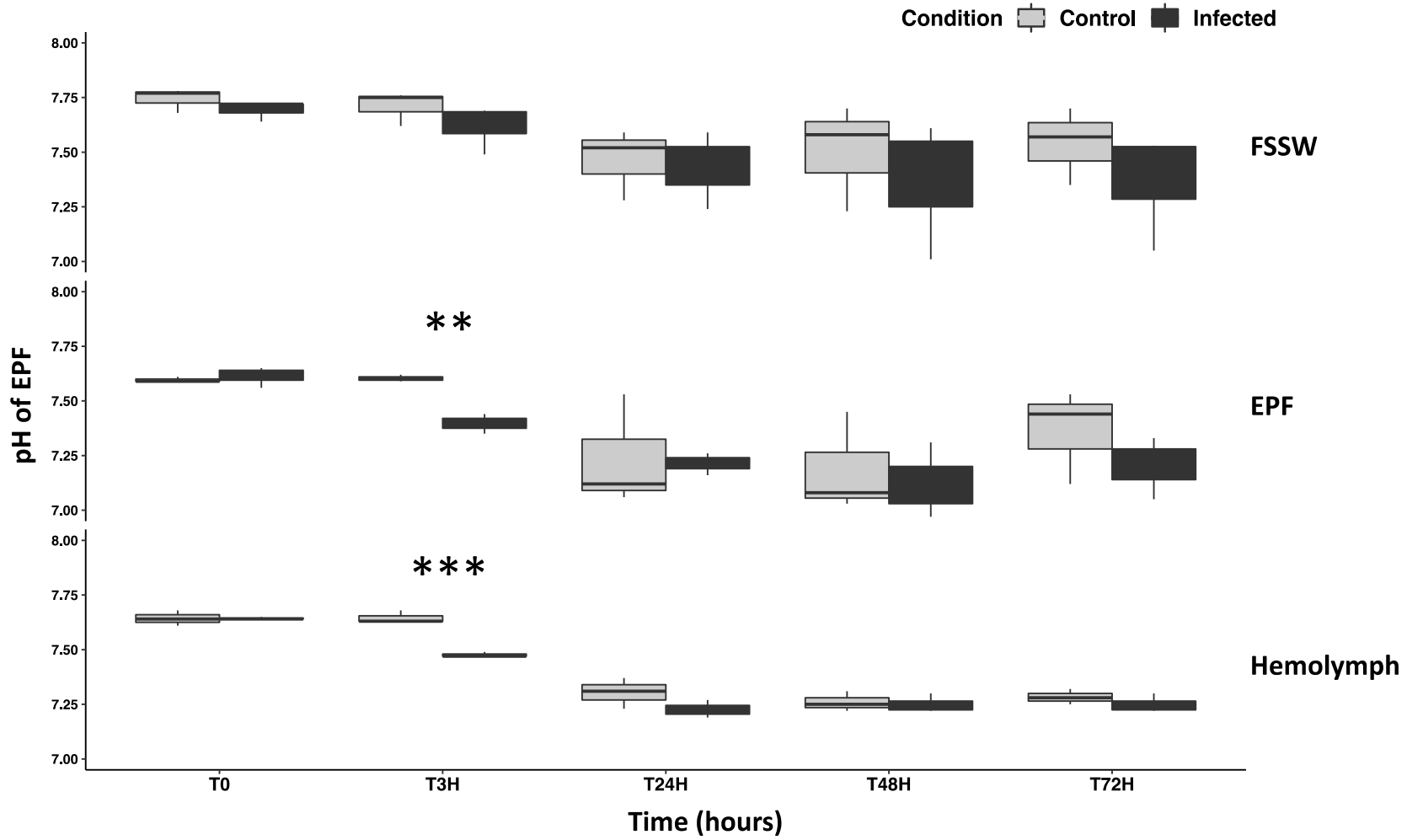

Figure 6: pH changes after *V. tapetis* exposure in FSSW (Filtered sterilized Seawater), EPF (extrapallial fluids) and Hemolymph (He) mixed with powdered Manila clam shell. ** : pvalue=0.0017 ; ***=pvalue=0.0007, Student test

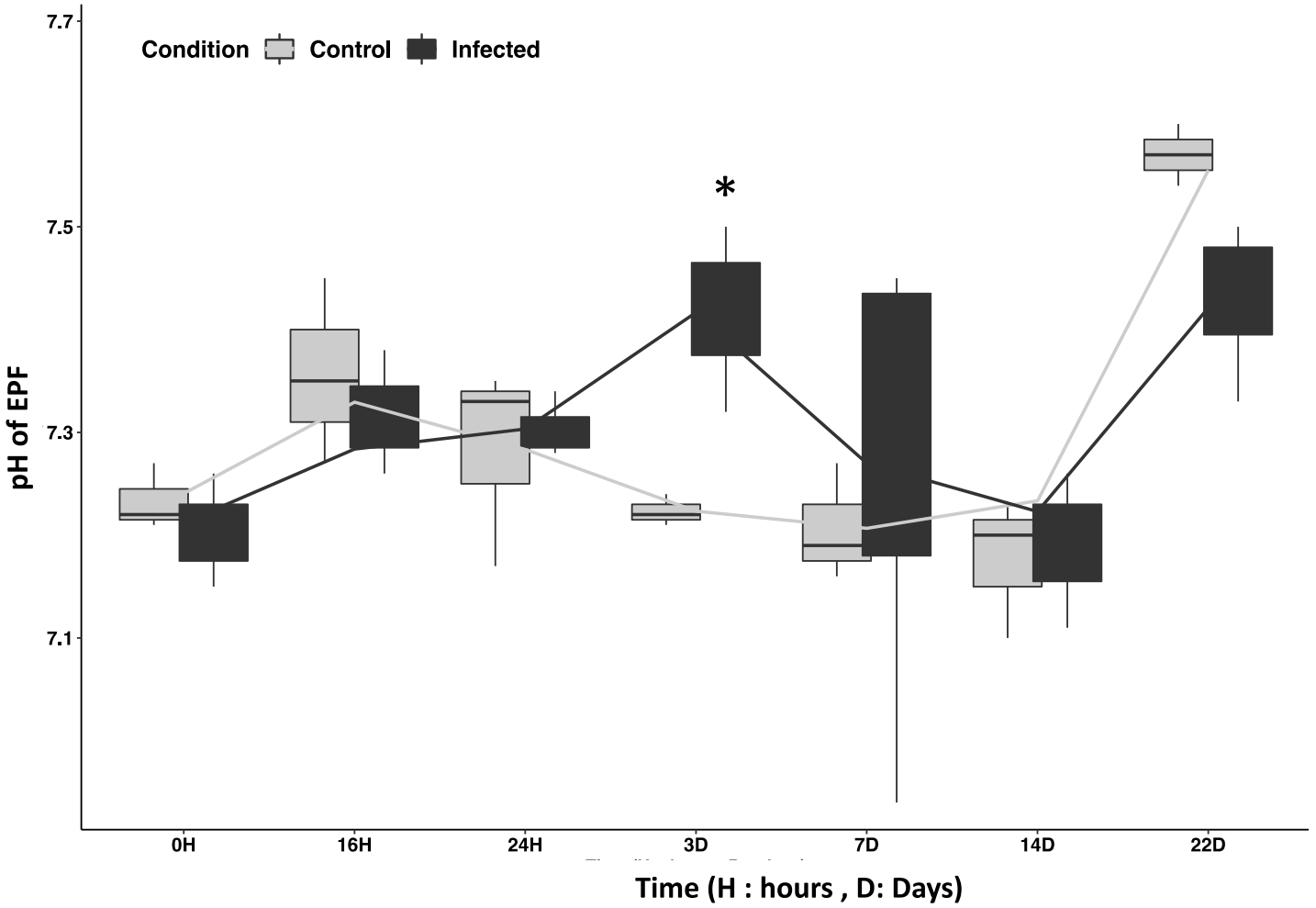

Figure 7: pH changes in EPFs (extrapallial fluids) of the Manila clam *Ruditapes philippinarum* after infection by *V. tapetis*. Control: injected by FSSW ; Infected: injected by *V. tapetis*

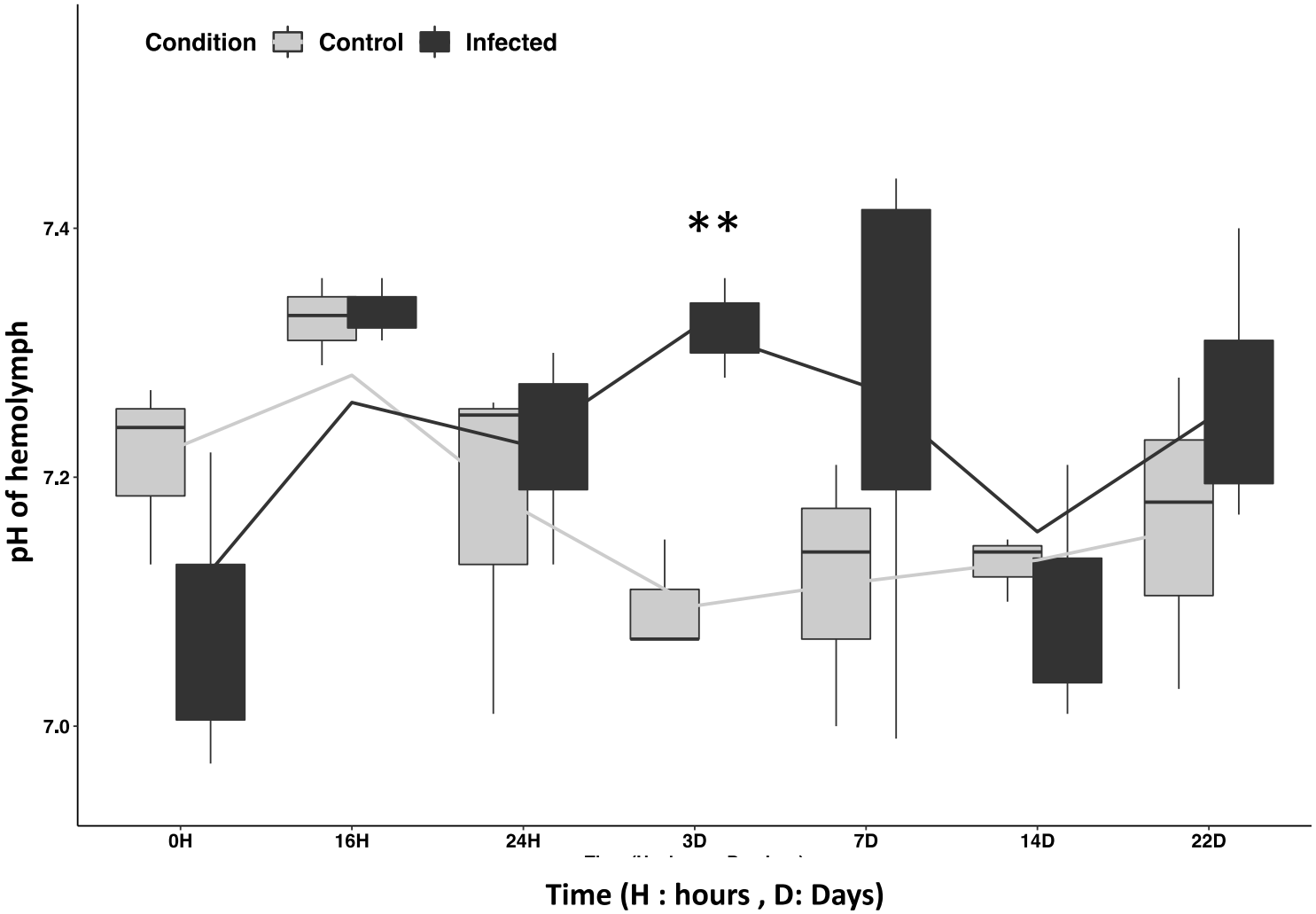

Figure 8: pH changes in Hemolymph of the Manila clam *Ruditapes philippinarum* after infection by *V. tapetis*. Control: injected by FSSW ; Infected: injected by *V. tapetis*

Table 1: growth parameters of *V. tapetis* in a range of pH media and different temperatures. DT : doubling Time ; OD : Optic Density, T° : Temperature; h: hour. M=Medium and the number is the pH (600= pH 6.00) ; MC is the original medium (unmodified pH).

		V. tapetis CECT4600 growth													
		M600		**M645**		**M700**		**MC**		**M786**		**M812**		**M870**	
T°	**Time of growth**	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}	DT (h)	Maximum OD_{492}
14°C	31H	5,00	0,945	3,31	1,121	3,39	1,155	3,15	1,047	3,50	1,128	3,16	1,143	7,03	0,946
18°C	24H	2,37	1,201	1,85	1,220	1,95	1,214	2,00	1,120	1,79	1,178	2,42	1,245	3,54	1,010
21°C	24H	2,19	1,231	1,93	1,204	2,30	1,207	2,50	1,167	2,83	1,201	2,52	1,223	3,97	1,061
27°C	24H	0	0,012	0	0,024	0	0,033	0	0,06	0	0,021	0	0,021	0	0,031