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## **External pH modulation during the growth of *Vibrio tapetis* , the aetiological agent of brown ring disease**

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

3

4 Running head: External pH modulations by *Vibrio tapetis*

5

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12

13 Abbreviations

14 BRD: Brown Ring Disease

15 d.p.i.: Days Post-Infection

16 EPFs: Extrapallial Fluids

17 FSSW: Filtered Sterilized Seawater

18 OD: Optical Density

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

21 **Abstract**

22 **Aims**

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

27 **Methods and Results**

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

34 **Conclusions**

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

42 **Significance and Impact of Study**

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

## 45 INTRODUCTION

46

47 The Manila clam *Ruditapes philippinarum* is a bivalve mollusc species of significant economic  
48 interest with a world production mainly from China, followed by European countries such as  
49 Italy, Spain and France (Paillard 2004b). It was imported in France in the early 70's for  
50 aquaculture. Ten years later, mortalities have been observed in clam's cultures, caused by an  
51 infection called the Brown Ring Disease or BRD (Paillard et al. 1989). Indeed, infected clams  
52 were characterized by a brown deposit in the inner face of the shell between the pallial line  
53 and the edge of the shell (Paillard et al. 1994). BRD is caused by a Gram negative bacterium,  
54 *Vibrio tapetis* (Paillard and Maes 1990; Borrego et al. 1996). BRD has caused significant  
55 economic losses in livestock farms on the Northern European coasts because the pathogen  
56 has an optimum infection rate in cold waters at around 14°C. For example, in France, Manila  
57 clam production decreased from 500 tons in 1987 to 200 tons in 1989, the aquaculture of  
58 clams production being mainly located in Brittany (France), an area highly sensitive to BRD  
59 (Paillard 2004b).

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

66

67 During infection by *V. tapetis*, the pathogen therefore colonizes the periostracum and  
68 degrades it in order to infiltrate peripheral or even central extrapallial fluids (EPFs). As a

69 result, it inhibits the biomineralization of the shell, preventing the formation of calcium  
70 carbonate crystals of aragonite and thus leads to the accumulation of the organic brown  
71 deposit (Paillard et al. 1994).

72 Furthermore, infected clams are facing an immunodepression caused by *V. tapetis*.  
73 Hemocytes, which are key effectors of the immune defenses of clams, are altered in BRD by  
74 actin cytoskeleton disorganization causing rounding of hemocytes and downregulation of  
75 pathogen recognition effectors. Consequently, infected hemocytes are no more able to  
76 ensure phagocytic progress to degrade the pathogen (Choquet et al. 2003; Allam et al. 2014;  
77 Rahmani et al. 2019).

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

84 Shell biomineralization is characterized by the formation of Calcium-Carbonate crystals  
85 ( $\text{CaCO}_3$ ) from calcium ( $\text{Ca}^{2+}$ ) and carbonate ( $\text{CO}_3^{2-}$ ) ions.  $\text{CaCO}_3$  crystals are the main  
86 constituent of shellfish. In the Manila clam, the shell is composed of aragonitic crystals  
87 (Taylor, J D et al. 1973; Trinkler et al. 2011). The saturation state of this mineral in seawater  
88 determines whether the biomineralization reaction is favored in the direction of crystal  
89 formation or dissolution. It is defined by the concentrations of  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  ions.

90 Biomineralization process is often studied in the context of ocean acidification because pH is  
91 a factor that considerably influences the physiology of marine calcified organisms, and  
92 especially this crystals formation. Indeed, acidification has the effect of reducing the

93 saturation rate of calcium carbonate crystals through the reduction in available  $\text{CO}_3^{2-}$  ions,  
94 thus promoting the dissolution of the shell (Gattuso and Hansson 2011). In the mussel *Mytilus*  
95 *edulis*, decrease in pH leads to a decrease in the net calcification rate,  $\text{Ca}^{2+}$  ion content and, in  
96 general, in a change in the ultrastructure of the shell and a decrease in amino acid content (Li  
97 et al. 2015). Combination of temperature and  $\text{pCO}_2$  increase results in a decreased shell  
98 hardness in the clam *Mercenaria mercenaria* and in the American oyster *Crassostrea virginica*,  
99 indicating major changes in the biomineralization process (Ivanina et al. 2013). In the brittle  
100 stars *Amphiura filiformis*, authors showed that the animal was able to compensate, in the  
101 short term, for the effects of pH decline by accelerating calcification and metabolism at the  
102 expense of certain functions such as growth and reproduction because of the high energy  
103 cost required for this mechanism (Wood et al. 2008). In summary, most of the studies reflect  
104 that ocean acidification reduces the calcification rates of animals composed of calcitic or  
105 aragonitic crystal shells by promoting the dissolution reaction of these crystals.

106 In the Manila clams, calcification, i.e. shell biomineralization reaction, occurs in extrapallial  
107 fluids: ie the mantle secretes the shell components, the organic matrix (conchiolin) and the  
108 calcium carbonate that form the aragonitic crystal shell (Paillard and Le Pennec 1993; Trinkler  
109 2009; Trinkler et al. 2011). The periostracum (outer part of the shell) is made of a sclerotized  
110 organic matrix. Shell organic matrix is mainly composed under normal conditions of aspartic  
111 acid, glycine and serine, responsible for binding with calcium ions: chelation by sulphate ester  
112 groups on polysaccharide chains would initiate biomineralization. When BRD develops,  
113 affected clams have deficiencies in amino acid such as glutamic acid, aspartic acid, serine,  
114 and alanine, which can lead to poor binding to Calcium - Carbonate (Gouletquer et al. 1989).  
115 **Given the importance of pH homeostasis in the biomineralization process, it seems likely**  
116 **that pH might play a significant role in the pathogenicity of *V. tapetis* in the case of BRD.**

117 Nevertheless, the influence of pH on the development of *V. tapetis*, as well as on the  
118 development of BRD, have never been investigated. In this paper, we chose to  
119 investigate the influence of pH on the development of the pathogen and the modulation  
120 of pH associated to this bacterium in clams' fluids. Furthermore, we monitored for the  
121 first time the pH modification in clam fluids during *in vivo* challenge by *V. tapetis* allowing  
122 us to formulate hypothesis that pH variations due to *V. tapetis* might be a virulence  
123 strategy of this pathogen linked to pathogenicity and to its ability to alters  
124 biomineralization. This study is the first investigation of pH modifications in the context  
125 of BRD.

126

## 127 **Materials and methods**

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

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

141 Growth experiments: colonies of strain CECT4600 were resuspended in 2 ml of Zobell medium  
142 (unmodified pH, control medium CM). The Optical Density (OD) was measured at a  
143 wavelength of 492 nm and the suspension concentration was adjusted to an OD of about 0.1,  
144 i.e. to a cell density of  $9.4 \times 10^7$  CFU.ml<sup>-1</sup>. The suspension was diluted at 1:100 in 3 ml of Zobell  
145 medium at the desired pH. The control samples were the same sterile media. 400 µL of  
146 inoculated media or controls were distributed in a Bioscreen© 100-wells plate (5 replicates  
147 per tested condition), allowing a sterile, thermally controlled culture with an OD  
148 measurement at regular intervals under stirring. This protocol was repeated twice; each  
149 experiment was run for 24 hours (except at 14°C where the experiment ran for 30 h to take



150 into account the reduced bacterial growth), in a shaking mode, with measurement of OD  
151 every 20 minutes at a wavelength of 492 nm ; temperature was set up at either 14, 18, 21 or  
152 27°C. To represents the pH measurement at the beginning of the growth experiment (To) and  
153 TF (TFinal) represent the pH measurement at the end of experiment, depending on the  
154 different conditions as detailed above.

155

156 pH monitoring during the bacterial growth: Two precultures of *V. tapetis* CECT4600 were  
157 performed in Zobell broth during 15 h at 18°C and 24 h at 14°C in order to reach an OD of  
158 0.98. Each preculture was diluted 12 times (initial OD = 0.08) with fresh Zobell medium at the  
159 different pHs (from 6.00 to 8.70). With these new cultures, bacterial growth was performed  
160 under shaking at 14°and 18°C, respectively from the corresponding pre-culture, in three  
161 replicates. The pH was measured, from 200 µl-samples, at the beginning of the experiment,  
162 every hour during the exponential growth phase, and finally in the stationary phase (i.e. 48 h  
163 of growth at 14°C and 24 h of growth at 18°C).

164

## 165 **2) Monitoring of the pH in extrapallial fluids (EPFs) and hemolymph of Manila** 166 **clams during infection by *V. tapetis***

167

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

172

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

183

184 For *in vitro* experiments, 150  $\mu$ l of FSSW, hemolymph or EPFs were placed in 1.5 ml sterile  
185 tubes, with supplementation of powdered Manila clam shell, in the same amount in each  
186 sample, in order to design an *in vitro* experiment that is as close as possible of natural  
187 conditions and to allow ion exchanges between fluids and shell that might be dependent of  
188 the *V. tapetis* growth. Then, 200  $\mu$ L of a *V. tapetis* CECT4600 suspension in FSSW was added  
189 to each tube, in order to reach a final bacteria:hemocytes ratio of 25:1 ( $1.37 \times 10^7$  CFU.ml<sup>-1</sup>);  
190 200  $\mu$ l of FSSW was added for the controls. Each condition was tested in triplicates. pH  
191 measures were performed at times 0 h, 3 h, 24 h, 48 h and 72 h and tubes were shaken before  
192 measurement.

193

194 For *in vivo* infection experiments, the protocol was followed as previously described (Paillard  
195 and Maes 1994). The animals were first left out of the water during the night before infection  
196 to allow the valves to open spontaneously in water, and then injected with 100  $\mu$ l of FSSW for

197 50 control individuals, or 100 µl of bacterial suspension ( $5 \times 10^8$  CFU.ml<sup>-1</sup> in FSSW) for 50  
198 infected individuals, in the pallial cavity. The injected animals were left out of water for 3  
199 additional hours to allow the pathogen to colonize them, and then were transferred again  
200 into their respective seawater tanks. Animals were sampled at different time points after  
201 infection, ie. after 15 hours, 1 day, 3 days, 7 days, 14 days and 21 days post injection (d.p.i.).  
202 pH of the EPFs and hemolymph samples were measured by using the Mettler Toledo  
203 microelectrode calibrated with pH 4, 7 and 9 buffers. The initial sample (To) was taken just  
204 before injection. The mode of infection does not induce mortality and allows to reproduce  
205 first stage of BRD to see the effect of *V. tapetis*, even before the development of organic  
206 deposit in the shell, highlighting the pH variations that occurs in the really first days of *V.*  
207 *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.

## 211 RESULTS

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

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

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

228 The pHs of the culture media have been measured at both the beginning (To) and the end  
229 (TF) of bacterial growth in all the conditions tested. Results are presented in Figures 1 to 3. In  
230 the control samples (no bacteria), the pHs did not change significantly between the  
231 measurements made at To and TF (Fig. 1 to 3). By contrast, after growth of *V. tapetis* at 14, 18  
232 and 21°C, the pH changed during growth to reach an average value of 7.90 in all media tested  
233 (Fig. 1 and 2). The pH modifications are the same at these three temperatures, allowing us to

234 show them all in the same figures (Fig. 1 and 2). We did not observe such a pH modification  
235 of the culture medium at 27°C, a condition where no growth of the bacterium was observed  
236 (Fig. 3).

237 Figure 2 shows the statistical distribution of pH values measured at To and TF in all media.  
238 The mean and variance of this distribution differed significantly during the experiment only  
239 when the bacterium was able to grow (*pvalue* < 0.0001). Overall, these results show that *V.*  
240 *tapetis* is able to modify the pH of its culture medium during its growth towards a pH close to  
241 7.90.

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

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

255 We aimed at determining whether the bacterium can also modulate the pH of clam  
256 biological fluids in which *V. tapetis* is known to proliferate during the infection, ie. the EPFs

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

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

273 The previous demonstration that the presence of *V. tapetis* can interfere *in vitro* with the pH  
274 of these clam biological fluids led us to hypothesize that this could happen *in vivo*, and that  
275 the modulation of pH of these fluids could be an important aspect of the infection process.  
276 Monitoring of pH evolution in EPFs and hemolymph after Manila clams infection by *V. tapetis*  
277 is presented in Figures 7 and 8, respectively. Both fluids displayed a relatively similar pH  
278 evolution following the injection of FSSW or *V. tapetis* in the animals. Considering the whole  
279 *in vivo* infection experiment (22 days), we did not observe any significant difference in pH  
280 between the control and infected animals for both fluids. However, we observed a weak but

281 significant difference between the pHs of the EPFs and hemolymph from control and infected  
282 clams at 3 days post injection (dpi). Indeed, the pH of these fluids were higher (of approx. 0.2  
283 pH unit) in both cases in clams injected with a suspension of *V. tapetis*, as compared to clams  
284 injected with FSSW (*pvalue* < 0.02). Our results then tend to suggest that exposure to *V.*  
285 *tapetis* induce physiological changes in challenged Manila clam that influence the pH of EPF  
286 and hemolymph at 3 days of exposure to the pathogen. This also suggests that this particular  
287 period should be more precisely investigated during the study of host-pathogen interaction.  
288

## 289 DISCUSSION

290 pH homeostasis is an essential aspect of cell physiology. It is also one of the main  
291 environmental factors controlling the biomineralization of calcified organisms. This explains,  
292 at least in part, the current abundance of studies dealing with the impact of ocean  
293 acidification on marine mollusks and other calcified marine organisms (Gattuso and Hansson  
294 2011). The relation between decrease of pH and an increase of temperature has been studied  
295 in several bivalves species, showing that these two factors can affect the immune response  
296 as in the Mediterranean mussel *Mytilus galloprovincialis* (Matozzo et al. 2012) and was also  
297 shown to influence the abundance of parasites and incidence of bacteria as in the blue mussel  
298 *Mytilus edulis* (Mackenzie et al. 2014). Finally, pH adaptation is a crucial component of  
299 virulence of many pathogenic bacteria. In addition to having to survive the acid digestive  
300 barriers in some hosts, they must be able to tolerate acid stress resulting from lysosomal  
301 activity after being phagocytized by immune system cells such as macrophages or hemocytes  
302 (Asplund et al. 2014).

303

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



313 We observed that *V. tapetis*, the bacterial etiological agent of BRD, is able to grow over a wide  
314 range of pHs, including at a cold temperature (14°C) which is an optimum for BRD  
315 development (Paillard et al. 2004). Interestingly, we showed that the bacterium, during its  
316 growth, modifies the pH of its environment, increasing it in acidic media, or acidifying it in  
317 alkaline ones, and systematically replaced the external pH to a value close to 7.7 during its  
318 growth in a kind of 'neutralizing activity' in the tested range. We further characterized this  
319 last point, by measuring *in vitro* the impact of the bacterial growth on the pH of the main  
320 biological fluids of the Manila clams, ie. extrapallial fluids and hemolymph, and then *in vivo*,  
321 the pH of these fluids in a context of infection by inducing the main mode of infection of *V.*  
322 *tapetis*, allowing the pathogen to colonize extrapallial fluids. We observed that at three hours  
323 after exposure to *V. tapetis*, the pH of the two fluids decreased significantly more than that  
324 of the control in presence of powdered Manila clam shell. EPFs and hemolymph contain  
325 hemocytes, which are the main cellular actors of the immune system and one of the main  
326 targets of the pathogen.

327

328 In a previous transcriptomic analysis, we showed that *V. tapetis* is able to induce  
329 deregulations of clam hemocytes physiology as reorganization of actin cytoskeleton,  
330 reduction of lysosomal activity and down regulation of genes related to the complement  
331 pathway (Rahmani et al. 2019). In another previous study, our team had demonstrated that  
332 the cytotoxicity of the bacterium to hemocytes was maximal, *in vitro*, after three hours of  
333 exposure to the pathogen (Choquet et al. 2003). This phenomenon is reproduced by exposing  
334 Manila clam hemocytes to *V. tapetis* during precisely three hours. The decrease in pH in the  
335 first three hours of exposure could be due to the growth of the pathogen and could be related

336 (either as a cause or a consequence) to its cytotoxic effect on clam hemocytes where the  
337 influence of the bacterium is maximum.

338

339 The relationship between pH regulation and virulence is not always clear since the regulation  
340 of the internal pH is a necessity for all cell types. Bacteria regulate their intracellular pH by  
341 using proton pumps, or by transporting and/or metabolizing several acid or base compounds.  
342 For example, *Escherichia coli* response to acid stress involves *amino acid decarboxylase*  
343 *antiporter pairs* and *proton-pumping respiratory chains complexes* causing H<sup>+</sup> ion efflux outside  
344 the cell. On the contrary, during alkaline stress, *E. coli* was found to have a strong activation  
345 of *NhaA Na<sup>+</sup>/H<sup>+</sup> antiporter*, an up-regulation of ATP synthetase leading to a strong entry of  
346 H<sup>+</sup> ion into the cell (Krulwich et al. 2011). However, intestinal bacteria are facing larger pH  
347 ranges than marine ecosystems. *NhaA* antiporter is also present in the genome of *V. tapetis*  
348 CECT4600 according to the recently published annotation (Dias et al. 2018), as well as  
349 calcium or potassium proton antiporters. Furthermore, the presence of an enzyme such as  
350 arginine deiminase in the genome of this pathogen might explain alkalinization of external  
351 pH observed during acidic stress as already shown in another bacterium (Budin-Verneuil et  
352 al. 2006).

353 Regardless of this internal pH regulation, many bacteria modify the pH of their environment  
354 as a result of their energy metabolism. This is particularly the case for many fermentative  
355 bacteria producing large quantities of organic acids (e.g. lactic acid, propionic acid, formic  
356 acid, etc.) leading to an acidification of their environment (Nuryana et al. 2019). Other  
357 bacteria are known to alkalinize their environment, by using organic acids (e.g. lactate,  
358 aspartate, glutamate) as carbon and energy sources (Stancik et al. 2002). Nevertheless, these  
359 metabolisms are very dependent on the nature of the substrates used by the bacteria, and

360 there are no case, to our knowledge, of bacteria capable of being both acidifying and  
361 alkalinizing in the same culture medium, only depending on the initial pH of that medium.  
362 This discovery raises many questions about the *V. tapetis*'s adaptation mechanisms to pH  
363 stress, as well as about the energy metabolism(s) it uses, and the substrates it degrades from  
364 the Zobell medium, at the different pHs. This also raises important questions regarding the  
365 involvement of this 'neutralizing activity' in the expression of its pathogenicity, in the context  
366 of BRD development. The mechanisms related to this activity are for now unknown and  
367 might be due to several type of mechanisms as described above. Nevertheless, this study has  
368 revealed new, previously unsuspected problematics related to pH modulation, highlighting  
369 the need for further analysis of genes expressed during growth in order to elucidate the  
370 mechanisms linked to this phenomenon.

371

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

384 periostracal lamina in order to enter extrapallial fluids, and then colonizes the shell secretion  
385 (Paillard and Maes 1995), leading to an increase of *V. tapetis* concentration in EPFs. Between  
386 2 days and 7 d.p.i. the EPFs concentration of *V. tapetis* reaches its maximum value (Bidault et  
387 al. 2015).

388 This particular period corresponds to an increase of pH in EPFs according to our study where  
389 significant differences of pH have been observed in infected animals after 3 days (*in vivo*). It  
390 should also be noted that an increase of pH has also been reported in hemolymph at 3 d.p.i.,  
391 thus probably revealing the changes that occurs in this compartment at the biochemical level  
392 during BRD (Allam et al. 2006).

393

394 In addition, we know that during infection, major changes occur in EPFs at the enzymatic  
395 level with, for example, an increase in the activity of phenoloxidase, an enzyme involved in  
396 the humoral response and melanization which leads to the brown deposit production  
397 (Söderhäll and Cerenius 1998). Previous studies showed that phenoloxidase activity is  
398 sensitive to abiotic factors such as pH (Le Bris 2013). Indeed, phenoloxidase activity decrease  
399 with pH in a range of physiological values in the Atlantic blue crab *Callinectes sapidus*, mostly  
400 in the hemolymph compartment (Tanner et al. 2006). Thus, increasing the pH in a range of  
401 physiological values in both hemolymph and EPFs could modulate phenoloxidase activity in  
402 these two compartments. As phenoloxidase is related to melanization process, the increase  
403 of pH can also modulate melanization, associated to the bacterial embedding within shell  
404 matrix.

405

406 Considering these informations, the fact that *V. tapetis* is able to modulate the pH of such  
407 compartments of the Manila clam in the context of BRD then questioned the impact of this

408 factor in the development of BRD itself. Indeed, pH is known to modulate the virulence of  
409 many pathogenic bacteria. In *Vibrio cholerae*, for example, it has been shown that the protein  
410 ToxR, which is responsible for the regulation of many virulence genes, is also strongly  
411 involved in the response to acid stress, suggesting a close relationship between the virulence  
412 and the acid stress response in this pathogen (Merrell and Camilli 2002; Lund et al. 2014). In  
413 *Pseudomonas aeruginosa*, it has been shown that the infection was attenuated at pH 6 rather  
414 than pH 7.6, and that the bacterium expresses genes that allow it to alkalinize the medium at  
415 acidic pH, while at neutral pH, it preferentially expresses iron metabolism-related genes  
416 (Romanowski et al. 2011). In the case of *V. tapetis*, we don't know yet if pH variations are due  
417 to sensing of external pH by *V. tapetis* that induce a change in response, maybe in relation  
418 with its pathogenic activity or to the growth of the pathogen. This mechanism should be  
419 investigated in a future study.

420

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

432 by both *in vitro* and *in vivo* challenges in order to better characterize these particular  
433 interactions and to understand the mechanisms and the importance of pH modulation in the  
434 pathogenic activity of *V. tapetis*.

435

436

#### 437 **AUTHORS CONTRIBUTIONS**

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

443

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449

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454

455 **CONFLICT OF INTEREST**

456 No conflict of interest declared.

457

458

459 **REFERENCES**

- 460 Allam B, Paillard C, Auffret M, Ford SE (2006) Effects of the pathogenic *Vibrio tapetis* on  
 461 defence factors of susceptible and non-susceptible bivalve species: II. Cellular and  
 462 biochemical changes following *in vivo* challenge. *Fish Shellfish Immunol* 20:384–397
- 463 Allam B, Pales Espinosa E, Tanguy A, Jeffroy F, Le Bris C, Paillard C (2014) Transcriptional  
 464 changes in Manila clam (*Ruditapes philippinarum*) in response to Brown Ring  
 465 Disease. *Fish Shellfish Immunol* 41:2–11 . <https://doi.org/10.1016/j.fsi.2014.05.022>
- 466 Asplund ME, Baden SP, Russ S, Ellis RP, Gong N, Hernroth BE (2014) Ocean acidification  
 467 and host–pathogen interactions: blue mussels, *Mytilus edulis*, encountering *Vibrio*  
 468 *tubiashii*. *Environ Microbiol* 16:1029–1039 . <https://doi.org/10.1111/1462-2920.12307>
- 469 Bidault A, Richard GG, Le Bris C, Paillard C (2015) Development of a Taqman real-time PCR  
 470 assay for rapid detection and quantification of *Vibrio tapetis* in extrapallial fluids of  
 471 clams. *PeerJ* 3:e1484 . <https://doi.org/10.7717/peerj.1484>
- 472 Borrego JJ, Castro D, Luque A, Paillard C, Maes P, Garcia MT, Ventosa A (1996) *Vibrio*  
 473 *tapetis* sp. nov., the causative agent of the brown ring disease affecting cultured  
 474 clams. *Int J Syst Evol Microbiol* 46:480–484
- 475 Budin-Verneuil A, Maguin E, Auffray Y, Ehrlich DS, Pichereau V (2006) Genetic structure  
 476 and transcriptional analysis of the arginine deiminase (ADI) cluster in *Lactococcus*  
 477 *lactis* MG1363. *Can J Microbiol* 52:617–622 . <https://doi.org/10.1139/wo6-009>
- 478 Choquet G, Soudant P, Lambert C, Nicolas J-L, Paillard C (2003) Reduction of adhesion  
 479 properties of *Ruditapes philippinarum* hemocytes exposed to *Vibrio tapetis*. *Dis*  
 480 *Aquat Organ* 57:109–116
- 481 Dias GM, Bidault A, Le Chevalier P, Choquet G, Der Sarkissian C, Orlando L, Medigue C,  
 482 Barbe V, Mangenot S, Thompson CC, Thompson FL, Jacq A, Pichereau V, Paillard C  
 483 (2018) *Vibrio tapetis* Displays an Original Type IV Secretion System in Strains  
 484 Pathogenic for Bivalve Molluscs. *Front Microbiol* 9:227
- 485 Gattuso J-Pierre, Hansson Lina (2011) Ocean acidification. Oxford University Press,  
 486 Oxford ;
- 487 Gouletquer P, Héral M, Béchemin C, Richard P (1989) Anomalies de calcification chez la  
 488 palourde japonaise *Ruditapes philippinarum*: caractérisation et comparaison des  
 489 compositions en acides aminés de différentes parties de la coquille analysées par  
 490 HPLC. *Aquaculture* 81:169–183
- 491 Ivanina AV, Dickinson GH, Matoo OB, Bagwe R, Dickinson A, Beniash E, Sokolova IM  
 492 (2013) Interactive effects of elevated temperature and CO<sub>2</sub> levels on energy  
 493 metabolism and biomineralization of marine bivalves *Crassostrea virginica* and  
 494 *Mercenaria mercenaria*. *Comp Biochem Physiol A Mol Integr Physiol* 166:101–111
- 495 Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and  
 496 homeostasis. *Nat Rev Microbiol* 9:330–343
- 497 Le Bris C (2013) Le système phénoloxydase: caractérisation biochimique et rôle dans la  
 498 réponse immunitaire chez la palourde japonaise *Venerupis philippinarum* exposée à  
 499 *Vibrio tapetis*. Université de Bretagne occidentale-Brest
- 500 Le Bris C, Richard G, Paillard C, Lambert C, Segueineau C, Gauthier O, Pernet F, Guérard F



501 (2015) Immune responses of phenoloxidase and superoxide dismutase in the manila  
502 clam *Venerupis philippinarum* challenged with *Vibrio tapetis* – Part I: Spatio-  
503 temporal evolution of enzymes' activities post-infection. *Fish Shellfish Immunol*  
504 42:16–24 . <https://doi.org/10.1016/j.fsi.2014.10.021>

505 Li S, Liu C, Huang J, Liu Y, Zheng G, Xie L, Zhang R (2015) Interactive effects of seawater  
506 acidification and elevated temperature on biomineralization and amino acid  
507 metabolism in the mussel *Mytilus edulis*. *J Exp Biol* 218:3623–3631 .  
508 <https://doi.org/10.1242/jeb.126748>

509 Lund P, Tramonti A, De Biase D (2014) Coping with low pH: molecular strategies in  
510 neutralophilic bacteria. *FEMS Microbiol Rev* 38:1091–1125 .  
511 <https://doi.org/10.1111/1574-6976.12076>

512 Mackenzie CL, Lynch SA, Culloty SC, Malham SK (2014) Future Oceanic Warming and  
513 Acidification Alter Immune Response and Disease Status in a Commercial Shellfish  
514 Species, *Mytilus edulis* L. *PLOS ONE* 9:e99712 .  
515 <https://doi.org/10.1371/journal.pone.0099712>

516 Matozzo V, Chinellato A, Munari M, Finos L, Bressan M, Marin MG (2012) First Evidence of  
517 Immunomodulation in Bivalves under Seawater Acidification and Increased  
518 Temperature. *PLOS ONE* 7:e33820 . <https://doi.org/10.1371/journal.pone.0033820>

519 Merrell D, Camilli A (2002) Acid tolerance of gastrointestinal pathogens. *Curr Opin*  
520 *Microbiol* 5:51–55 . [https://doi.org/10.1016/S1369-5274\(02\)00285-0](https://doi.org/10.1016/S1369-5274(02)00285-0)

521 Nuryana I, Andriani A, Lisdiyanti P, Yopi (2019) Analysis of organic acids produced by lactic  
522 acid bacteria. *IOP Conf Ser Earth Environ Sci* 251:012054 .  
523 <https://doi.org/10.1088/1755-1315/251/1/012054>

524 Paillard C, Allam B, Oubella R (2004) Effect of temperature on defense parameters in  
525 Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. *Dis Aquat*  
526 *Organ* 59:249–262

527 Paillard C (2004a) A short-review of brown ring disease, a vibriosis affecting clams,  
528 *Ruditapes philippinarum* and *Ruditapes decussatus*. *Aquat Living Resour* 17:467–475 .  
529 <https://doi.org/10.1051/alr:2004053>

530 Paillard C (2004b) Rôle de l'environnement dans les interactions hôtes-pathogènes;  
531 développement d'un modèle de vibriose chez les bivalves. *Habilit À Dir Rech HDR*  
532 *Univ Bretagne Occident Brest*

533 Paillard C (2016) An ecological approach to understanding host-pathogen-environment  
534 interactions: the case of Brown Ring Disease in clams. In: *Oysters and Clams:*  
535 *Cultivation, Habitat Threats and Ecological Impact*

536 Paillard C, Le Pennec M (1993) Ultrastructural studies of the mantle and the periostracal  
537 lamina in the manila clam, *Ruditapes philippinarum*. *Tissue Cell* 25:183–194 .  
538 [https://doi.org/10.1016/0040-8166\(93\)90018-G](https://doi.org/10.1016/0040-8166(93)90018-G)

539 Paillard C, Maes P (1990) Aetiology of brown ring disease in *Tapes philippinarum*:  
540 pathogenicity of a *Vibrio* sp. *Comptes Rendus Académie Sci Ser 3 Sci Vie* 310:15–20

541 Paillard C, Maes P (1994) Brown ring disease in the Manila clam *Ruditapes philippinarum*:  
542 establishment of a classification system. *Dis Aquat Organ* 19:137–146

543 Paillard C, Maes P (1995) The Brown Ring Disease in the Manila Clam, *Ruditapes*  
544 *philippinarum*. Part 1. Ultrastructural alterations of the periostracal lamina. J  
545 Invertebr Pathol 65:91–100 . <https://doi.org/10.1006/jipa.1995.1015>

546 Paillard C, Maes P, Oubella R (1994) Brown ring disease in clams. Annu Rev Fish Dis 4:219–  
547 240 . [https://doi.org/10.1016/0959-8030\(94\)90030-2](https://doi.org/10.1016/0959-8030(94)90030-2)

548 Paillard C, Percelay L, Le Pennec M, Le Picard D (1989) Origine pathogène de l'«anneau  
549 brun» chez *Tapes philippinarum* (Mollusque, bivalve). Comptes Rendus Académie  
550 Sci Sér 3 Sci Vie 309:235–241

551 Rahmani A, Corre E, Richard G, Bidault A, Lambert C, Oliveira L, Thompson C, Thompson  
552 F, Pichereau V, Paillard C (2019) Transcriptomic analysis of clam extrapallial fluids  
553 reveals immunity and cytoskeleton alterations in the first week of Brown Ring  
554 Disease development. Fish Shellfish Immunol 93:940–948 .  
555 <https://doi.org/10.1016/j.fsi.2019.08.025>

556 Romanowski K, Zaborin A, Fernandez H, Poroyko V, Valuckaite V, Gerdes S, Liu DC,  
557 Zaborina OY, Alverdy JC (2011) Prevention of siderophore- mediated gut-derived  
558 sepsis due to *P. aeruginosa* can be achieved without iron provision by maintaining  
559 local phosphate abundance: role of pH. BMC Microbiol 11:212 .  
560 <https://doi.org/10.1186/1471-2180-11-212>

561 Söderhäll K, Cerenius L (1998) Role of the prophenoloxidase-activating system in  
562 invertebrate immunity. Curr Opin Immunol 10:23–28 .  
563 [https://doi.org/10.1016/S0952-7915\(98\)80026-5](https://doi.org/10.1016/S0952-7915(98)80026-5)

564 Stancik LM, Stancik DM, Schmidt B, Barnhart DM, Yoncheva YN, Slonczewski JL (2002)  
565 pH-Dependent Expression of Periplasmic Proteins and Amino Acid Catabolism in  
566 *Escherichia coli*. J Bacteriol 184:4246–4258 . [https://doi.org/10.1128/JB.184.15.4246-  
567 4258.2002](https://doi.org/10.1128/JB.184.15.4246-4258.2002)

568 Tanner CA, Burnett LE, Burnett KG (2006) The effects of hypoxia and pH on phenoloxidase  
569 activity in the Atlantic blue crab, *Callinectes sapidus*. Comp Biochem Physiol A Mol  
570 Integr Physiol 144:218–223

571 Taylor, J D, Kennedy, W J, Hall, A (1973) The Shell Structure and Mineralogy of the Bivalvia.  
572 II. *Lucinacea-Clavagellacea*. Conclusions. Bull Br Mus Nat Hist Zool Lond 22:253–  
573 294

574 Trinkler N (2009) La guérison coquillière: un mécanisme de défense de la palourde  
575 japonaise *Ruditapes philippinarum* face au *Vibrio tapetis* dans le cadre de la maladie  
576 de l'anneau brun. Université de Bretagne occidentale-Brest

577 Trinkler N, Bardeau J, Marin F, Labonne M, Jolivet A, Crassous P, Paillard C (2011) Mineral  
578 phase in shell repair of Manila clam *Venerupis philippinarum* affected by brown ring  
579 disease. Dis Aquat Organ 93:149–162 . <https://doi.org/10.3354/dao02288>

580 Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification  
581 rates, but at a cost. Proc R Soc Lond B Biol Sci 275:1767–1773

582 Zobell C (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic  
583 aerobes. J Mar Res 4:41–75

584

## FIGURES AND TABLES

Figure 1: pH changes in a <i>V. tapetis</i> 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).....	27
Figure 2: pH variance of a <i>V. tapetis</i> 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 .....	28
Figure 3: pH changes in a <i>V. tapetis</i> CECT4600 culture after growth at 27° in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) (15 replicates) .....	29
Figure 4: pH change in a culture of <i>V. tapetis</i> 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. ....	30
Figure 5: pH change in a culture of <i>V. tapetis</i> 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. ....	31
Figure 6: pH changes after <i>V. tapetis</i> 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 .....	32
Figure 7: pH changes in EPFs (extrapallial fluids) of the Manila clam <i>Ruditapes philippinarum</i> after infection by <i>V. tapetis</i> . Control: injected by FSSW ; Infected: injected by <i>V. tapetis</i> .....	33
Figure 8: pH changes in Hemolymph of the Manila clam <i>Ruditapes philippinarum</i> after infection by <i>V. tapetis</i> . Control: injected by FSSW ; Infected: injected by <i>V. tapetis</i> .....	34
Table 1: growth parameters of <i>V. tapetis</i> 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). ....	35

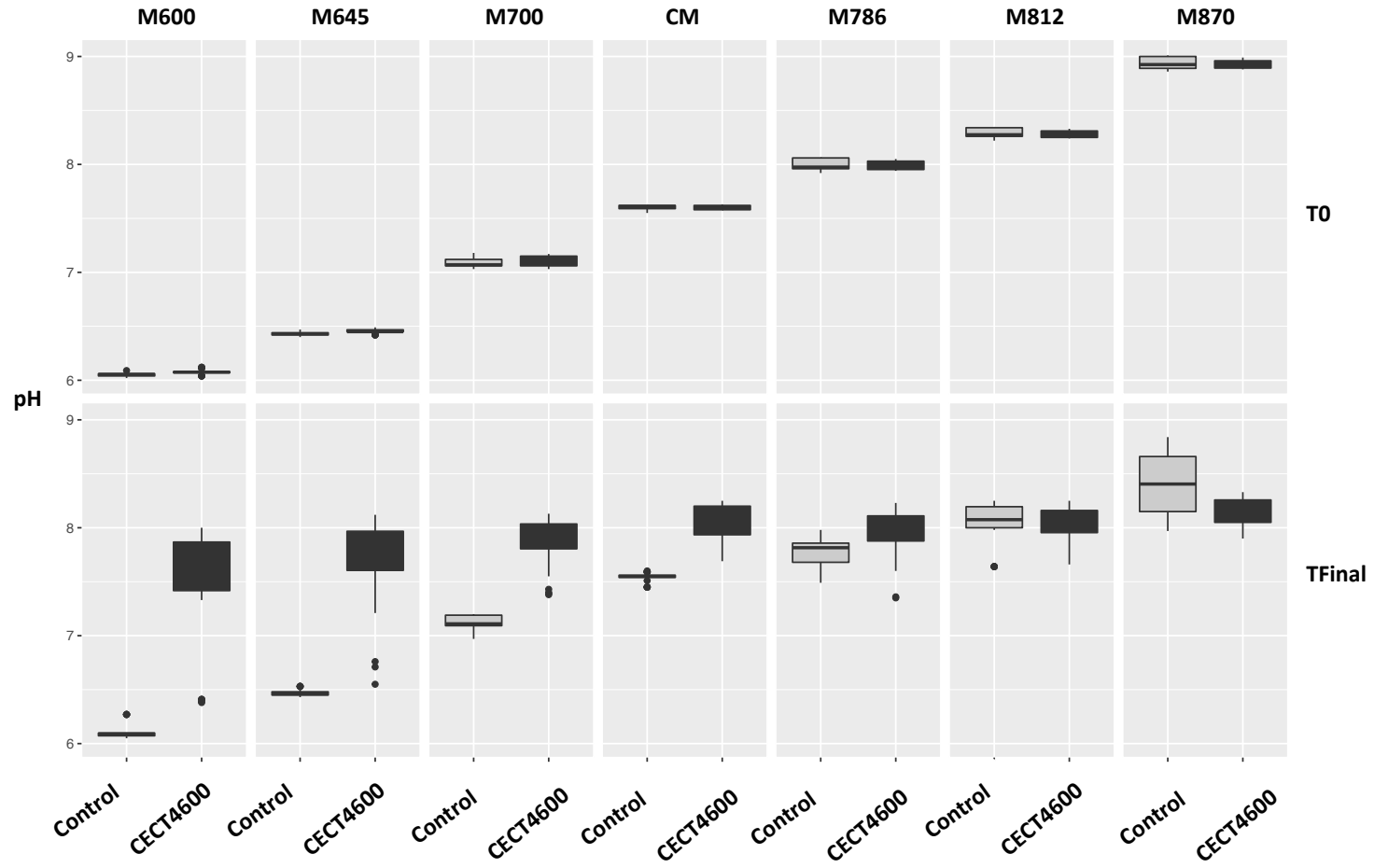


Figure 1: pH changes in a *V. tapetis* CECT<sub>4600</sub> culture after growth at 14°C, 18° and 21°C in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) (15 replicates)

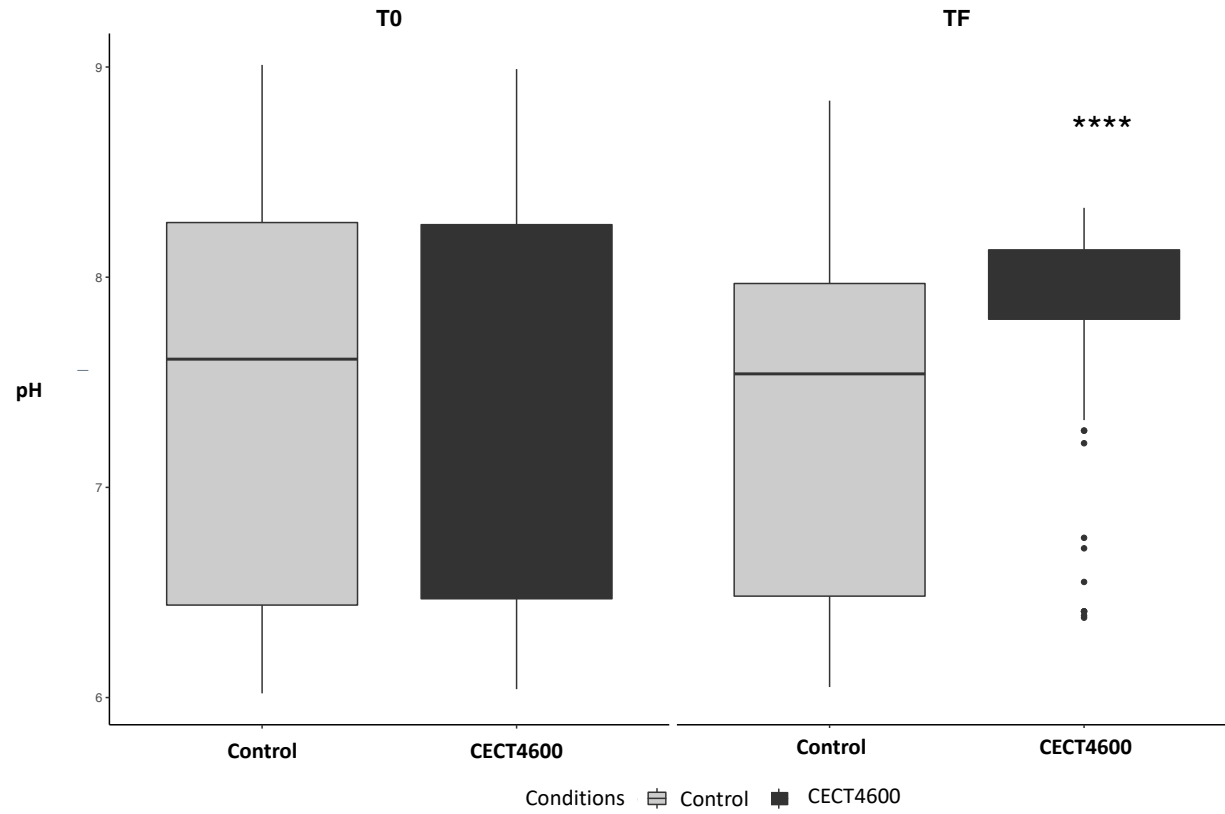


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\*\*\*\* : pvalue < 0.0001, Student test

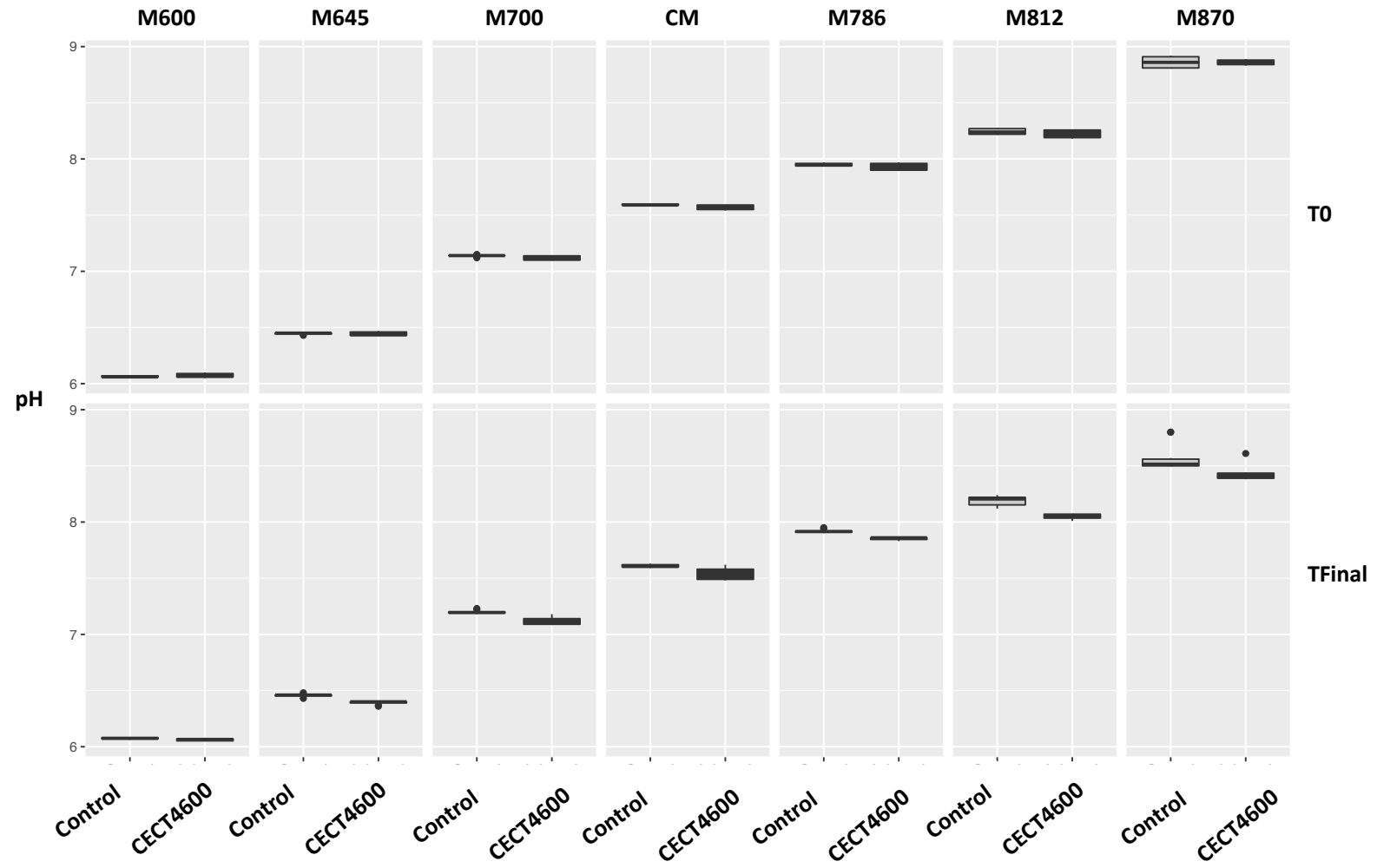


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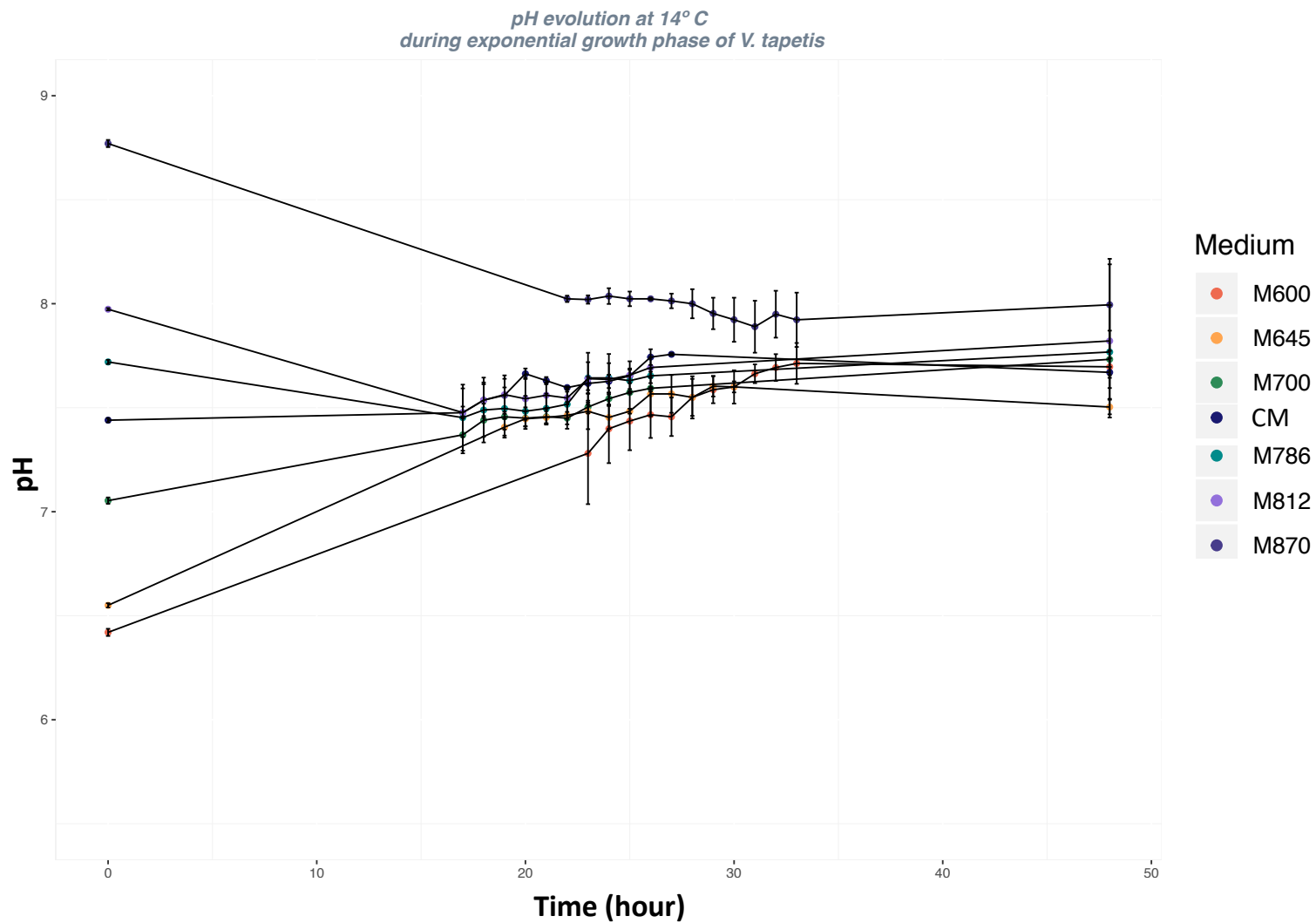


Figure 4: pH change in a culture of *V. tapetis* during the exponential growth phase at 14°C, in Zobell media which pH were initially adjusted from 6.00 (M600) to 8.70 (M870) ; CM is the original medium where pH was not modified.

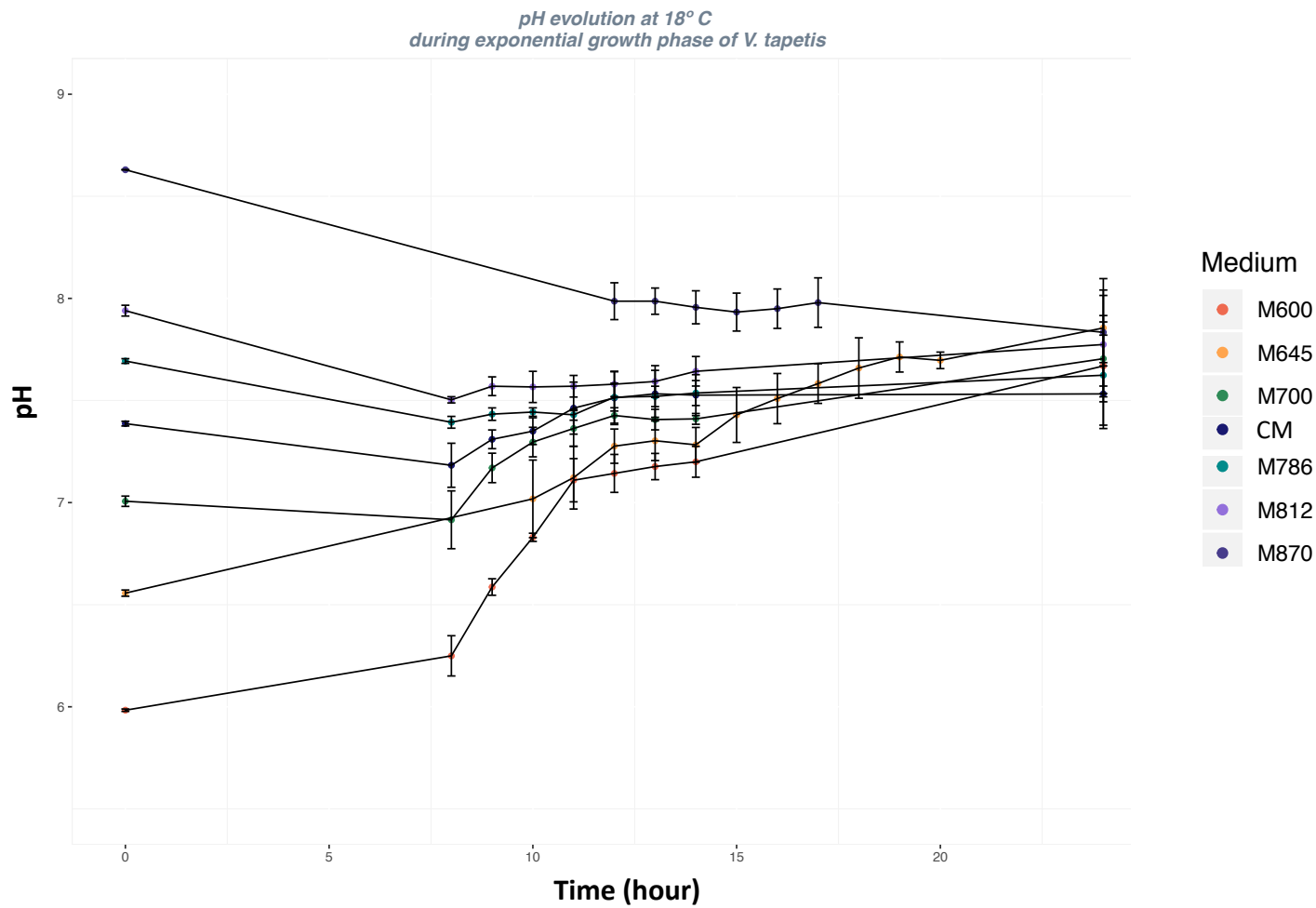


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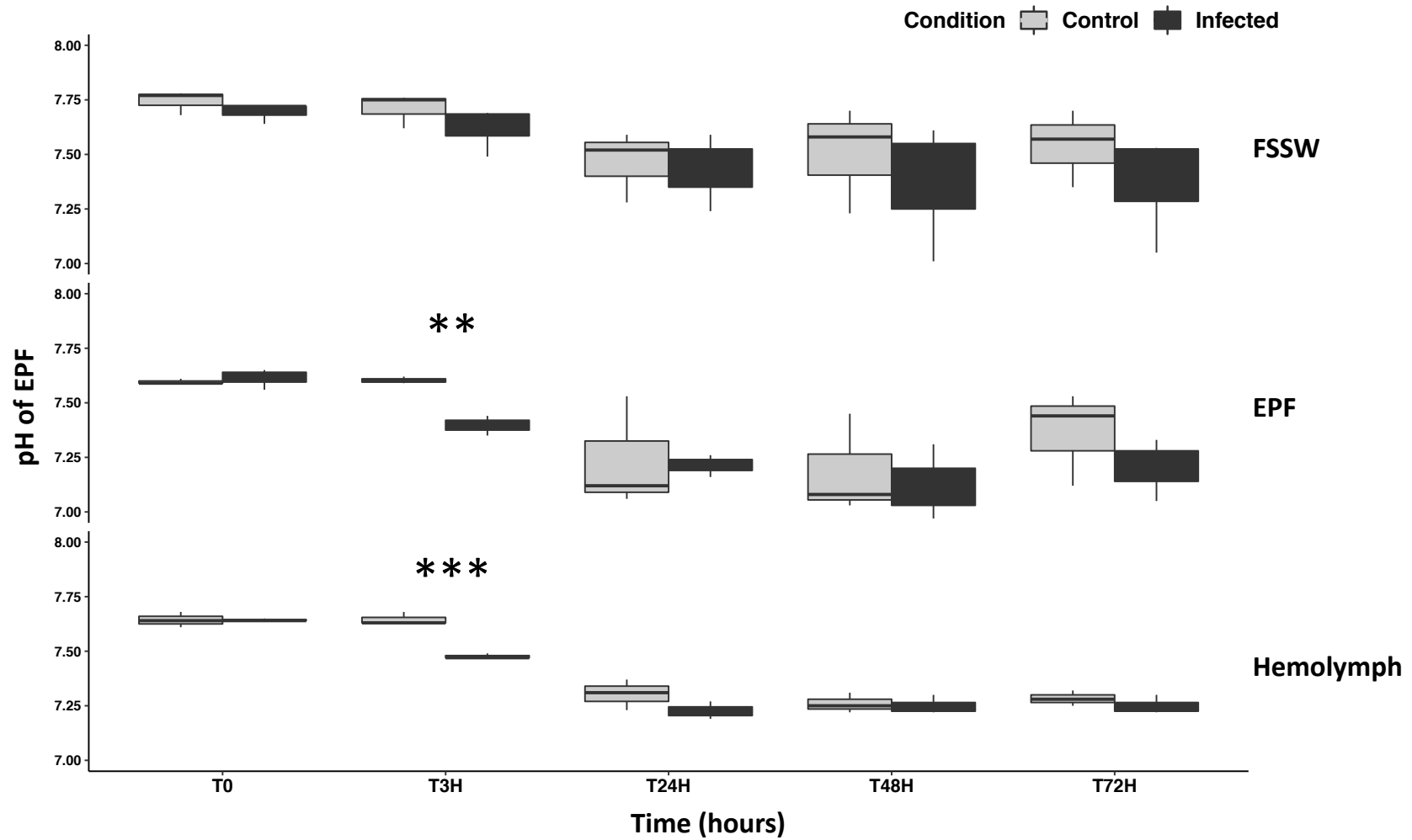


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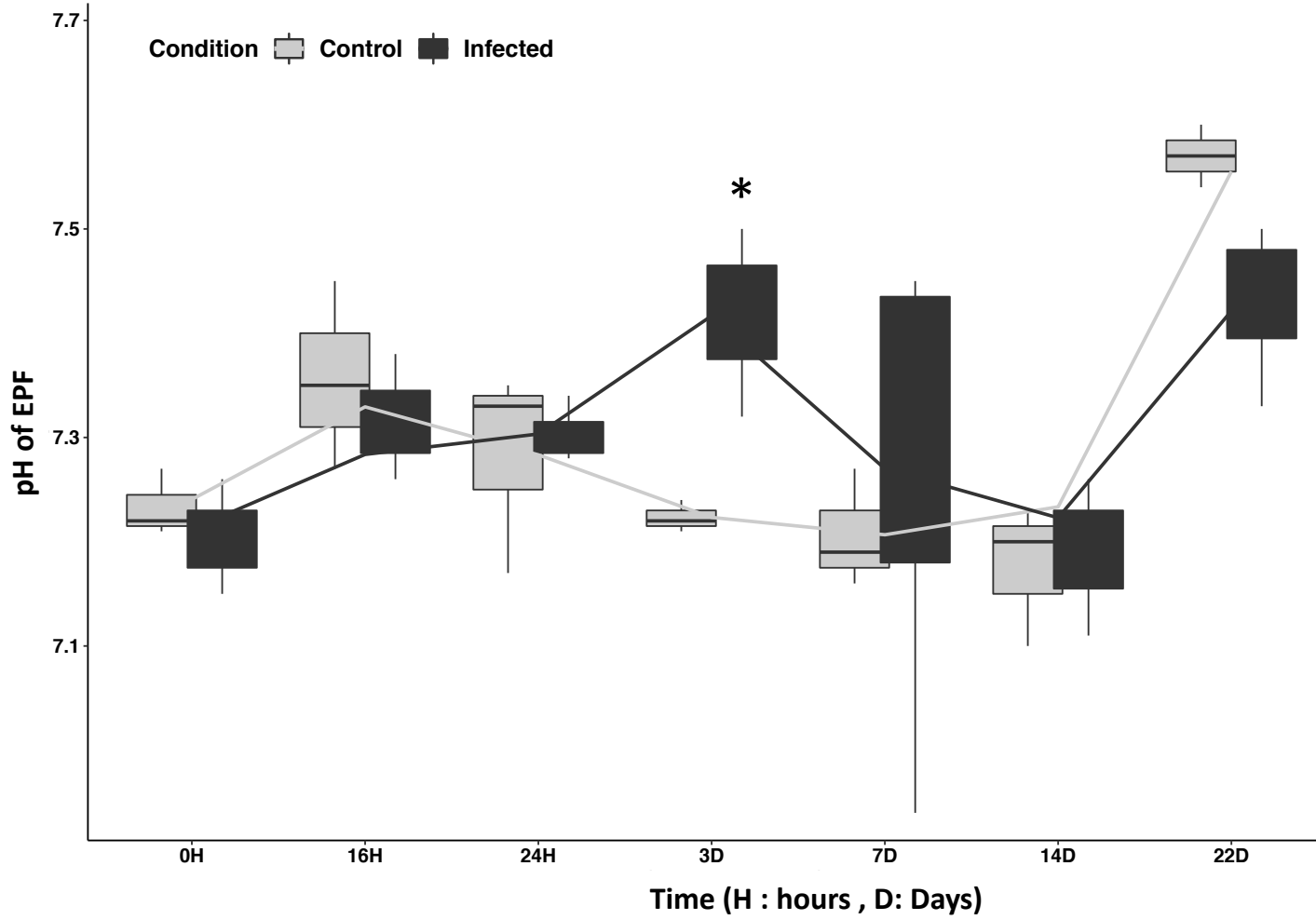


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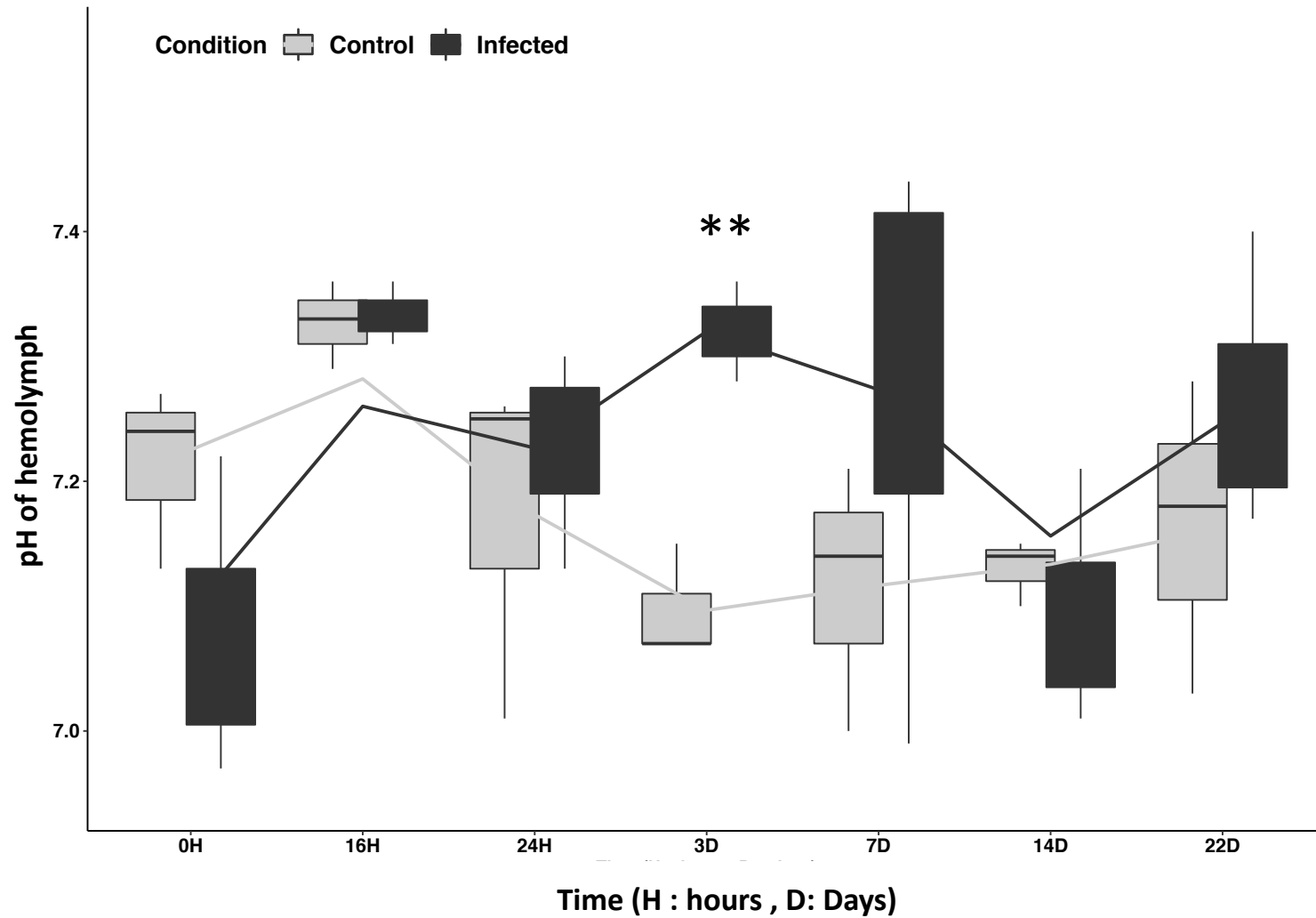


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<i>V. tapetis</i> CECT4600 growth															
T°	Time of growth	M600		M645		M700		MC		M786		M812		M870	
		DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>	DT (h)	Maximum OD <sub>492</sub>
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