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### ► To cite this version:

Alexandra Rahmani, Erwan Corre, Gaelle Richard, Adeline Bidault, Christophe Lambert, et al.. Transcriptomic analysis of clam extra pallial fluids reveals immunity and cytoskeleton alterations in the first week of Brown Ring Disease development. *Fish and Shellfish Immunology*, 2019, 93, pp.940-948. <10.1016/j.fsi.2019.08.025>. <hal-02409065>

**HAL Id: hal-02409065**

**<https://hal.science/hal-02409065v1>**

Submitted on 9 Sep 2020

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## Transcriptomic analysis of clam extra pallial fluids reveals immunity and cytoskeleton alterations in the first week of Brown Ring Disease development

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### Abstract :

The Brown Ring Disease is an infection caused by the bacterium *Vibrio tapetis* on the Manila clam *Ruditapes philippinarum*. The process of infection, in the extrapallial fluids (EPFs) of clams, involves alteration of immune functions, in particular on hemocytes which are the cells responsible of phagocytosis. Disorganization of the actin-cytoskeleton in infected clams is a part of what leads to this alteration. This study is the first transcriptomic approach based on collection of extrapallial fluids on living animals experimentally infected by *V. tapetis*. We performed differential gene expression analysis of EPFs in two experimental treatments (healthy-against infected-clams by *V. tapetis*), and showed the deregulation of 135 genes. In infected clams, a downregulation of transcripts implied in immune functions (lysosomal activity and complement- and lectin-dependent PRR pathways) was observed during infection. We also showed a deregulation of transcripts encoding proteins involved in the actin cytoskeleton organization such as an overexpression of  $\beta$ 12-Thymosin (which is an actin sequestration protein) or a downregulation of proteins that closely interact with capping proteins such as Coactosin, that counteract action of capping proteins, or Profilin. We validated these transcriptomic results by cellular physiological analyses that showed a decrease of the lysosome amounts and the disorganization of actin cytoskeleton in infected hemocytes.

## Graphical abstract



## Highlights

► During BRD, most of regulated transcripts are involved in the immune system, translation and actin cytoskeleton organization. ► We showed a downregulation of the immune system related to the complement pathway through C1q and lectin during infection. ► We highlighted a deregulation of transcripts involved in actin organization related to capping proteins. ► Physiological validations of immune and actin deregulation were performed. ► This study opens new perspectives to explore pathogenicity in the case of BRD.

**Keywords** : Brown Ring Disease, *V. tapetis*, *R. philippinarum*, Hemocytes, Actin cytoskeleton, b-thymosin, Coactosin, Resting cells

36 **Abbreviations**

37 ABP: Actin Binding Protein

38 BRD: Brown Ring Disease

39 EPF: Extrapallial Fluids

40 FSW: Filter Sterilized Seawater

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## 1. INTRODUCTION

45 *Ruditapes philippinarum*, the Manila clam, has been imported in Europe from Indo-  
46 pacific coasts in the early 70's. Ten years later, a disease causing a brown organic  
47 deposit at the inner face of the shell, between the pallial line and the edge of the shell,  
48 spread mostly all over Northern coasts of Europe, causing mass mortalities to this  
49 species [1,2]. This infection has been called the Brown Ring Disease (BRD, according to  
50 its macroscopic manifestations) and is due to the bacterium *Vibrio tapetis* [3,4].

51 *V. tapetis* is a Gram-negative bacillus that acts, in most of cases, as an external  
52 microparasite, mainly in cold waters (optimal infection temperature of 14°C [5]) by  
53 spreading into the extrapallial fluids (EPF) of the Manila clam. Despite the fact that  
54 growth declines and shell deformations are frequently observed in this disease, tissue's  
55 infections are rare and only happen in case of pre-existing tissues lesions, then causing  
56 the death of animals. Nevertheless, BRD is a microparasitic infection which dynamics  
57 does not imply the death of animals [4,6].

58 A particular symptom of BRD is the rounding of hemocytes in infected clams, thus  
59 revealing damages to the clam's immune system [7,8]. Indeed, hemocytes are key  
60 effectors of the immune defenses of the clam that eliminate pathogens by phagocytosis  
61 [9]. In a normal phagocytosis process, hemocytes spread their pseudopods which  
62 encapsulate and internalize bacteria and, by a phagosome/lysosome fusion, destroy the  
63 pathogen. During BRD infection, it was shown that *V. tapetis* causes a disorganization of  
64 the hemocyte actin cytoskeleton, resulting in the loss of hemocytes pseudopods (and  
65 thus, the rounding of cells) thus reducing its phagocytosis capacity [10]. *V. tapetis* is  
66 then able to multiply into hemocytes, finally resulting in cell membrane disruption.

67 Bacteria can then spread into clams before being released by pseudo feces into the  
68 environment (sediments) where they can infect other clams [7,11–13].

69 This study is the first transcriptomic approach based on collection of extrapallial fluids  
70 (EPF) on living animals experimentally infected by *V. tapetis*. This study led to the  
71 identification of a set of transcripts regulated during BRD infection, combined with  
72 physiological experiments performed in order to check the validity of some of the major  
73 interpretations raised from the differential expression analysis. This study gave new  
74 insights to understand the pathogenicity of *V. tapetis* to Manila clams.

75

## 76 2. MATERIALS AND METHODS

### 77 2.1. Clams *in vivo* infection, RNA extraction and sequencing

78 Animals and experimental design are fully described in [14]. Two years-old clams were  
79 harvested in the Marennes-Oleron Bay (Charente Maritime, France) on May 5th, 2014.

80 A first visual health diagnostic was performed on 50 clams *in situ* and no BRD clinical  
81 signs were observed. Clams were then transferred to Ifremer facilities in Plouzané  
82 (Finistère, France) and held in a 6-day quarantine with a chloramphenicol (8 mg.L<sup>-1</sup>)  
83 treatment to avoid local contamination. After quarantine, animals were randomly divided  
84 and maintained in trays placed in eighteen 100 L-tanks equipped with air-lift systems  
85 (100 clams per tank). Water temperature was maintained at 13°C during the whole  
86 experiment so that infection by *V. tapetis* was favored [5]. Tanks were continuously  
87 supplied with filter sterilized seawater (FSW, filtered at 1 µm and UV-sterilized) at a  
88 renewal rate of 50% per day and cleaned every two days. Clams were fed *ad libitum*  
89 with T-iso. Manila clams, *R. philippinarum*, were injected in the extrapallial cavity with  
90 100 µL of *V. tapetis* CECT4600 suspension at the concentration of 10<sup>7</sup> CFU.mL<sup>-1</sup>

91 (Infected, 900 clams) or by Filter Sterilized Seawater (FSW, Control, 900 clams) as  
92 described in Le Bris et al [15]. At 7 days post injection, EPFs of infected and control  
93 clams were harvested and total RNAs were extracted with TRI reagent® according to  
94 the procedure manufacturer (Sigma-Aldrich®). Presence or absence of the Brown Ring  
95 Disease have been diagnosed according to the standardized methodology established  
96 by Paillard et al [16] based on the presence and localization of brown deposit in clam  
97 shells. We performed this diagnosis by an image analysis method using a 50-mm  
98 CANON macroscopic lens and analyzed using Visilog 6.6 image analysis software,  
99 according to image analysis methodology already performed [14,16]. This method  
100 allows to visualize the shell and to analyze the presence of a brown deposit (BRD  
101 positive diagnosis) with an image analysis software. BRD was diagnosed in all infected  
102 clams whereas no BRD was found in control animals. As ribosomal RNA (rRNA) is the  
103 most abundant component in total RNA, while not being informative in this kind of  
104 RNAseq strategy, we performed two rRNA depletion steps, the first one targeting  
105 bacterial rRNA and the second one targeting eukaryotic ones (by using the Ribo-Zero  
106 rRNA Removal Kits, bacteria and Human/Mouse/Rat, respectively, from Illumina). The  
107 RNAs from seven clams, 3 healthy and 4 infected, were subjected to cDNA libraries  
108 creation according to standard Illumina procedures (TruSeq Stranded mRNA Library  
109 Prep Kit). All the libraries were sequenced, by Illumina Miseq PE300, at the Federal  
110 University of Rio de Janeiro, Brazil. Other control and infected clams have been further  
111 analyzed for other questions [14].

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## 115 2.2. Transcriptomic analysis

116 Raw data analysis and differential expression evaluation were carried out using the  
117 ABIMS Galaxy platform, Station Biologique de Roscoff, CNRS / Sorbonne Université,  
118 France (<http://galaxy.sb-roscoff.fr>, January 2019).

119 Reads have been trimmed using AdaptorRemoval v2 [17], Trimmomatic v0.36.3 [18]  
120 and Prinseq v0.20.4 [19] in order to remove read adaptors, low quality sequences and  
121 poly(A) tails. Persistent rRNA were removed with SortMeRNA v2.1b.4 [20]. For the  
122 differential analysis, all the reads were mapped to a dataset of 61 747 transcripts (built  
123 by Arnaud Tanguy, Station Biologique de Roscoff – CNRS, Sorbonne University,  
124 France) obtained from a combination of *R. philippinarum*'s stimulated hemocytes ESTs  
125 (expressed sequences tag) library [21], from *R. philippinarum*'s ESTs deposited in NCBI  
126 EST database (<https://www.ncbi.nlm.nih.gov/nucest/>) and sequences deposited in the  
127 Ruphibase database (<http://compgen.bio.unipd.it/ruphibase/>). Transcripts abundances  
128 were estimated by using the “Align and Build expression matrix” pipeline (mapping with  
129 bowtie + reads estimation with RSEM) of the Trinity suite v2.4.0.2 [22]. The statistical  
130 analysis of differentially expressed transcripts were done using the “Differential  
131 expression analysis” pipeline of Trinity suite v2.4.0 (using DESeq2) and “Extract and  
132 cluster differentially expressed transcripts” pipeline of Trinity suite v2.4.0 [22]. We have  
133 set up parameters to exclude differential expression with a LogFoldChange between -2  
134 and 2. Transcripts were considered as significantly differentially expressed if the p-value  
135 adjusted by FDR (false discovery rate) is  $<10^{-3}$ . The identified deregulated transcripts  
136 were subjected to blast2GO for re-annotation, including blastX vs the NCBI nr, refseq  
137 and swissprot (2018 version) non redundant databases, interpro, GO mapping and GO  
138 annotation, all with standard parameters. All the annotations and functions were

139 carefully manually checked. The sequencing data have been made available at the  
140 European Nucleotide Archive (project PRJEB23385). The analysis data are given in  
141 Table S1.

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### 143 2.3. Clams *in vitro* infection : bacterial exposure

144 Animals used in this study were Manila clams from the SATMAR shellfish aquaculture  
145 site in Marennes (Charente-Maritime, France). The clam pool was acclimatized in  
146 oxygenated seawater using a bubbler at 14°C. Hemolymph was harvested directly from  
147 the adductor muscle. Each hemolymph was sampled individually and the quality of the  
148 hemocytes present in this fluid was checked by observation under the microscope  
149 (presence of pseudopods, few round-shaped hemocytes). Good quality hemolymph  
150 samples were pooled and the hemocytes enumerated by using a Malassez cell. For  
151 exposure assays, hemolymph was exposed to a bacterial suspension (in FSSW) of *V.*  
152 *tapetis* CECT4600, at a 25/1 : bacteria/hemocyte ratio. Hemocytes exposure were  
153 performed in at least 3 replicates and two independent experiments for each condition  
154 tested. Briefly, 100 µL of hemolymph was added in 24-well plates. After a few minutes,  
155 in order to let hemocytes attach on the bottom of the plate, 100 µL of bacterial  
156 suspension was added for the exposed samples, and 100 µL of FSSW for the controls.

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#### 158 2.3.1. F-actin visualization by microscopy

159 To visualize F-actin in control and infected hemocytes, we used the fluorescent probe  
160 Rhodamine Phalloidin (Invitrogen). Exposure assays were performed as described  
161 above. After 3 hours of challenge, we collected the supernatant and added 30 µL of  
162 Triton X-100 0.1% for 15 minutes. Triton permeabilizes cells, thus allowing probes to

163 enter the cell. 2  $\mu$ L of Rhodamine-phalloidin (Stock solution dissolved in 1.5 mL  
164 methanol) was then added to each sample (hemolymph non exposed and exposed to *V.*  
165 *tapetis*) which were stained for 20 min, on ice, without light. Epifluorescence microscopy  
166 was then performed by using a ZEISS AXIO observer Z1 inverted microscope with a  
167 wavelength of excitation BP (band pass) 550/25 nm, Beam splitter 570 nm and of  
168 emission BP 605/70 nm. Cells were observed both in red epifluorescence and in bright  
169 field.

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### 171 2.3.2. LysoTracker assay

172 The LysoTracker® probes (Molecular Probes ®) are fluorescent acidotropic probes for  
173 labeling and tracking acidic organelles in live cells, according to the manufacturer  
174 definition. We have developed and tested a method to characterize the amount of acidic  
175 organelles on hemocytes exposed to *V. tapetis* or FSSW for control. LysoTracker assay  
176 is performed during the *in vitro* bacterial exposure assay based on the rounding of  
177 hemocytes caused by *V. tapetis* previously developed by Choquet et al. [7]. Our protocol  
178 allows to perform both tests on the same sample, and then to correlate the rounding  
179 phenotype to the cell content in acidic organelles.

180 Hemocytes exposure were performed as described above. The 24 wells plates were  
181 incubated 1 hour at 18°C before addition of 4  $\mu$ L of 50  $\mu$ M LysoTracker® Red DND-99  
182 (Invitrogen, emission from 550 to 700nm, final concentration 1  $\mu$ M) to each well. The 24  
183 wells plates were then let 2 hours more, in dark conditions at 18°C, to reach the 3 hours  
184 exposition. The contents of the wells were then transferred in 5-mL cytometry  
185 polystyrene tubes (Falcon®, BD Biosciences, San Jose, CA, USA).

186 First, flow cytometry analyses were performed by using a BD FACSVerser flow cytometer  
187 using its blue laser (488 nm) as an excitation source. The mean red fluorescence level  
188 (LysoTracker fluorescence linked to acidic organelles) of the selected hemocytes was  
189 measured using the PerCP-Cy5.5 detector of the flow cytometer (700/54 nm).

190 Second, 2  $\mu$ L of a 100X dilution in ultra pure water of a commercial solution of SYBR-  
191 Green® I nucleic acid gel stain 10 000X in DMSO (Life Technology, USA) were added in  
192 each cytometry tube for 10 min, at room temperature, in dark condition, before a second  
193 flow cytometry analysis. Hemocytes were selected according to their green fluorescence  
194 (SYBR-Green, FITC detector of the flow cytometer 527/32 nm). Addition of SYBR-Green  
195 also allowed to quantify the number of non-adherent hemocytes in our sample.

196 This second flow cytometry analyses of red fluorescence were performed using a red  
197 laser (640 nm) as an excitation source and fluorescence was measured using the APC  
198 detector of the flow cytometer (660/10 nm). This detector was selected instead of the  
199 PerCP-Cy5.5 detector (700/54 nm) to overcome a possible SYBR-Green fluorescence  
200 overlapping. Results are expressed in mean red fluorescence level per hemocyte in  
201 arbitrary units (UA).

202

### 203 3. RESULTS AND DISCUSSION

#### 204 3.1. Transcriptomic results: differential expression analysis

205

206 The total number of reads was 29.3 million, with an average length of 262.5 bp, for a  
207 total coverage of 5.62X. Differential expression analysis revealed 135 differentially  
208 expressed genes, which 90 and 45 appeared down- and up- regulated, respectively, in  
209 the EPF treatment (*V. tapetis* infected clams), as compared to the control treatment. We

210 could obtain an annotation, and functional information, for 118 of them, which were  
211 classified according to their probable cellular role (Table S1, Table 1). In all, 85% of  
212 these transcripts belonged to four functional categories, ie. translation (34 transcripts),  
213 cytoskeleton (27), immune response (24) and metabolism (15) (Table 1). The  
214 deregulation of these transcripts in clams EPFs appears clearly related to the infection  
215 by *V. tapetis*. In the following section, we discuss the pathogen induced modulation of  
216 the immune response, which is a major point to better understand the host-pathogen  
217 interactions. Table 2 summarizes all the differentially expressed transcripts involved in  
218 cytoskeleton or immunity. It is noteworthy that the whole reads were also mapped  
219 against the recently released genome of *V. tapetis* CECT 4600 [23], but no significant  
220 correspondence has been found with the pathogen.

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233 Table 1: Number and functions of the transcripts differentially expressed in clams EPFs during *V. tapetis*  
234 infection  
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FUNCTIONS	Total number of transcripts	DOWN REGULATED IN INFECTED CLAMS	UP REGULATED IN INFECTED CLAMS
Translation	34	23	11
Cytoskeleton	27	18	9
Immunity	24	18	6
Metabolism	15	9	6
Proteins processing	6	4	2
Unknown / Hypothetical / Other	29	18	11

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238 Table 2: Transcripts significantly regulated during infection by *V. tapetis* and implied in the clam immune  
 239 system or in actin cytoskeleton organization. FDR : false discovery rate, LogFC : Log Fold-Change.  
 240

DOWN REGULATED IN INFECTED CLAMS			UP REGULATED IN INFECTED CLAMS		
Seq. Description	logFC	FDR	Seq. Description	logFC	FDR
<b>Immune system</b>			<b>Immune system</b>		
cathepsin K-like	-10.52	1.25E-05	<i>complement C1q-like protein 4</i>	11.02	4.29E-04
cathepsin K	-10.04	4.87E-04	cyclophilin-like protein	9.45	3.93E-04
cathepsin I	-9.93	4.16E-04	lysozyme	9.16	4.87E-04
cathepsin L1	-9.81	3.31E-04	Cyclophilin A	7.60	8.56E-05
cathepsin I	-9.77	2.27E-11	Dermatopontin	6.29	8.88E-04
saposin b domain-containing protein	-9.42	1.79E-11	cyclophilin-like protein	4.91	9.48E-04
<i>saposin B domain-containing protein precursor</i>	-8.74	2.95E-09			
cell death-inducing p53-target protein 1	-8.46	2.95E-09			
cathepsin S	-8.22	2.50E-05			
ferric-chelate reductase 1	-8.08	4.99E-04			
CD63 antigen	-7.51	1.44E-04			
defense protein 3-like	-7.30	5.66E-04			
<i>complement C1q-like protein 4</i>	-7.24	4.50E-05			
galectin-3 isoform 1	-7.20	6.22E-04			
c1q domain containing protein 1q13	-6.89	1.83E-06			
complement c1q TNF-related protein 3-like isoform 2	-6.34	3.82E-07			
complement c1q-like protein 2 precursor	-5.76	2.63E-04			
saposin b domain-containing protein	-5.04	1.25E-05			
<b>Actin cytoskeleton organization</b>			<b>Actin cytoskeleton organization</b>		
actin	-11.30	1.49E-04	actin	12.46	7.50E-05
actin	-11.21	8.94E-05	cytoplasmic actin	10.82	1.46E-04
actin	-10.49	5.02E-05	actin	10.82	1.49E-04
cytoplasmic actin	-10.18	4.81E-08	<i>Kinesin</i>	10.33	4.34E-08
Myophilin	-10.12	1.98E-05	<i>actin</i>	8.84	9.99E-04
cytoplasmic actin	-10.09	5.50E-05	thymosin beta-12	8.76	4.93E-04
coactosin-like protein	-9.73	2.07E-05	actin	8.57	6.14E-04
beta-actin	-8.86	1.27E-04	cytoplasmic actin	8.34	6.65E-04
cytoplasmic actin	-8.30	2.95E-09	myosin light chain	5.57	1.98E-05
actin 5c	-7.96	4.99E-04			
cytoplasmic actin	-7.45	4.99E-04			
cytoplasmic actin	-7.37	7.39E-04			
coactosin-like protein	-6.94	4.93E-04			
cytoplasmic actin	-6.84	2.82E-07			
cysteine and glycine-rich protein 3	-6.80	5.97E-04			
profilin	-6.63	9.08E-04			
cysteine and glycine-rich protein 3	-6.63	8.28E-04			
dynein light chain, cytoplasmic	-6.52	9.99E-04			

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## 246 3.2. Immune response

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248 Extrapallial fluids (EPFs) play an essential role in the biomineralization of the shell of  
249 bivalves. Given the particular etiology and symptoms of BRD, this compartment,  
250 containing numerous hemocytes [24], is particularly interesting to better understand both  
251 the infection process and how the clam's hemocytes fight against it. For this reason, in  
252 this study, *V. tapetis* infection was carried out directly inside the EPFs passing through  
253 the periostracal lamina barrier to accelerate the immune response, particularly in EPFs.  
254 A transcript encoding lysozyme was found to be upregulated in response to *V. tapetis*  
255 infection. In previous studies, lysozyme activities in cell lysates of extrapallial fluids were  
256 significantly higher 7 days after *V. tapetis* inoculation into the pallial cavity, as compared  
257 to control ones [24]. The antibacterial protein lysozyme, which is circulating in body  
258 fluids such as hemolymph and EPFs, is an important line of defense of the animal  
259 against bacterial pathogens because of their bacteriolytic properties [5,25].

260

261 Only five transcripts directly related to immunity were actually upregulated during *V.*  
262 *tapetis* infection. Beside the lysozyme, three genes belonging to the cyclophilin family,  
263 as well as dermatopontin, were upregulated (Table 2). Dermatopontin is a tyrosine-rich  
264 acidic matrix protein (TRAMP) that primarily acts as a structural component of the  
265 extracellular matrix. However, this protein was shown to modulate TGF- $\beta$  activity as well  
266 as cell-cell aggregation during infection in invertebrates [26]. Dermatopontin was  
267 previously shown to be upregulated in the EPFs and hemolymph of clams during BRD  
268 [27]. Not less than 3 cyclophilin transcripts appeared upregulated following *V. tapetis*  
269 infection. Cyclophilin was shown to be transcriptionally upregulated during infection in

270 the shrimp [28]. In the clam *R. philippinarum*, Chen et al. [29] previously showed that  
271 two cyclophilin genes were differentially expressed in hemolymph when exposed to  
272 pathogenic bacteria.

273  
274 The cellular component of the innate immune system also includes more specific  
275 pathways that involve the recognition of specific Pathogen-Associated Molecular  
276 Patterns (PAMPs) by pattern recognition receptor (PRRs). Most of these receptors are  
277 membrane proteins (eg. Toll like receptors), but they also include soluble proteins (eg.  
278 SAP, CRP) that are activated by the C1q proteins of the complement system [30–32].  
279 When the pathogen is recognized by either C1q proteins or Mannose Binding Proteins  
280 (MBP), these proteins activate the complement pathway through the “classical pathway”  
281 or the “lectin pathway” resulting in phagocytosis with the formation of a phagosome.  
282 [30]. Pathogens are then degraded after the fusion of the phagosome and the lysosome  
283 by the action of low pH, acid hydrolases and by reactive oxygen species produced  
284 during the pathogen-induced oxidative burst [33].

285  
286 In this study, 18 out of the 24 deregulated genes potentially involved in clams’ immune  
287 defenses were downregulated. Nine of these transcripts encoded proteins clearly related  
288 to lysosome activity (ie., cathepsins, saposins), four were C1q complement proteins,  
289 while others encoded CD63 (a cell death-inducing P53-target protein), galectin 3, as well  
290 as two proteins annotated as Defense Proteins, that should be related to ferric iron  
291 chelation/reduction. Different transcripts of C1q proteins were also reported in the clam  
292 *R. decussatus* infected with *Perkinsus olseni* [34], and in *R. philippinarum* infected with  
293 *V. tapetis* [27].

294  
295 Pathogens have developed many strategies to bypass the immune defenses of their  
296 hosts. In the particular case of BRD, it has been shown that the immune system of  
297 clams is not able to eliminate *V. tapetis* during infection, even if very high phagocytic  
298 rates of *V. tapetis* were measured when the pathogen is injected into the extrapallial  
299 space [35]. However, during phagocytosis by hemocytes, *V. tapetis* could persist and  
300 multiply within hemocytes and was also able to inhibit the phagosome-lysosome fusion,  
301 thus preventing its destruction [6]. The repression of nine genes encoding saposins (2)  
302 and cathepsins (7) could reflect the ability of the bacteria to inactivate the lysosomal  
303 functioning. Indeed, Cathepsins K, L, L1 and S are cysteine proteases [36][37] that  
304 belong to lysosomal acid hydrolases, that are normally released during the phagosome-  
305 lysosome fusion. Saposins are small lysosomal proteins that participate  
306 (stimulate/activate) to the hydrolysis of sphingolipids and many other lipids [38].  
307 Interestingly, the CD63 antigen belongs to the family of lysosomal associated proteins  
308 (LAMPs) and is also considered as an indicator of the phagosome-lysosome fusion [39].  
309 In all, these results strongly suggest that lysosomal function plays a key role in the  
310 development of BRD.

311 In addition to the predominant role of lysosome inactivation, the repression of genes  
312 encoding three complement C1q/tumor necrosis factor proteins, and of the galectin 3,  
313 might suggest an important role for the complement- and lectin-dependent PRR  
314 pathways [40,41]. The downregulation of a cell death-inducing P53-target protein, which  
315 is known to regulate the TNF-alpha-mediated apoptosis, might also suggest a role for  
316 apoptotic processes during the infectious process [42].

317 Finally, the downregulation of a protein annotated as 'defense protein', that contains a  
318 ferric-chelate reductase domain, could reflect an important role for iron acquisition  
319 during the infectious process. Interestingly, the concomitant downregulation of ferritin,  
320 which is implied in iron storage [43] and reduction of reactive oxygen species (ROS)  
321 accumulation [44], could also be related to this, allowing then more availability of iron for  
322 the pathogen.

323

### 324 3.3. Ribosomal proteins

325 We also identified deregulated genes involved in functions other than immunity, but  
326 whose deregulation could strongly influence the host's immune response. This is  
327 especially the case for cytoskeleton reorganization, thoroughly discussed thereafter, but  
328 also for translation proteins, which is the most modified category and, in other ways, for  
329 genes encoding proteins which function was categorized in metabolism and protein  
330 processes (Table 1).

331 A major reorganization of the translational apparatus may have occurred in response to  
332 *V. tapetis* infection as a result of gene expression reprogramming (32 ribosomal proteins  
333 and 2 translation elongation factors). Eleven genes were induced, while 23 genes had  
334 their expression reduced (Table 1). Ribosome inactivation resemble one of the major  
335 symptoms of the so-called 'metabolic depression' observed in many environmental  
336 stress responses in several marine mollusks [45,46]. However, it is noteworthy that the  
337 expression of several ribosomal proteins was increased during infection (S14, S21, S30,  
338 L21, SA, P0), and others appeared both repressed and induced (L3, L8, L27). For these  
339 last proteins, we presume that the protein exists as different alternate isoforms (with  
340 different regulations).

341 It is noteworthy that ribosomal proteins are increasingly studied for their alternate roles,  
342 ie. independent of their ribosomal function [45]. More specifically, it should be noted that  
343 the role of ribosomal proteins in the regulation of the innate immune response has been  
344 well documented in recent years [45], especially the major role of the ribosomal protein  
345 L13A in the GAIT complex, that selectively modulates the translation of some genes  
346 involved in the interferon- $\gamma$ -mediated inflammatory response [47]. However, many other  
347 examples have been reported. For example, the ribosomal proteins S14 and L8, both  
348 found upregulated in our study, would be able to activate P53 and the major regulator  
349 NF- $\kappa$ B, respectively [48,49]. It would be particularly interesting to perform new focused  
350 investigations to better understand the role of the translation apparatus in the  
351 development of the BRD.

352

353

#### 354 3.4. Actin cytoskeleton organization

355 Actin filaments (F-actin) belong to the cell cytoskeleton and are dynamically polymerized  
356 and depolymerized from monomers of actin (G-actin) into the cell to enable cellular  
357 activities such as locomotion, exocytosis or phagocytosis. The actin cytoskeleton is also  
358 responsible for the cell shape. In this study, we showed that at least 27 transcripts  
359 involved in actin cytoskeleton organization were differentially expressed during infection,  
360 among which 9 were induced while 18 had their expression reduced. Genes  
361 downregulated during infection are mainly involved in actin filament stabilization such as  
362 myophilin, a member of calponin protein family [50,51], cysteine glycine rich protein  
363 (CSRP) 1 [52,53] and dynein light chain [54]. Furthermore, our results showed that

364 expression regulation during infection also affected transcripts involved in cell motility  
365 such as up regulation of the Myosin light chain, which role in hemocytes phagocytosis in  
366 shrimp was already described [55].

367

368 Two transcripts downregulated during BRD might give additional information on the  
369 molecular mechanisms related to infection. The first one encodes a Coactosin like  
370 protein that counteracts the action of capping proteins that reduce or slow down actin  
371 polymerization [56]. The second one encodes Profilin, an 'actin binding protein' (ABP)  
372 which is known to interact with others ABPs such as  $\beta$ 4-thymosin [57]. The role of  
373 profilin in actin polymerization is quite complex. In the presence of molecules that  
374 stimulate actin assembly, profilin displaces  $\beta$ 4-thymosin, bounds to G-actin and  
375 promotes actin polymerization [58]. Furthermore, profilin regulates the effects of capping  
376 proteins. Hopmann and Miller [59] demonstrated that balanced activities of capping  
377 protein and profilin are essential in the regulation of actin dynamics and organization in  
378 *Drosophila*.

379 Capping proteins can be associated, in resting cells, with actin filament by electrostatic  
380 interactions on the barbed (+) end. By changing the conformation of the capping protein  
381 during this interaction, the + end is no longer available for G-actin and polymerization is  
382 then inhibited [60,61].

383

384 In this study, we also highlighted an overexpression of  $\beta$ 12 thymosin transcripts during  
385 infection by *V. tapetis*.  $\beta$ 12-thymosin belongs to the ABPs family. Fifteen variants of  $\beta$ -  
386 thymosin proteins have been described in many vertebrates and invertebrates, but not

387 on prokaryotes and yeasts. They are able to bind ATP-G-actin complex (1:1), the  
388 monomeric form of actin, and stabilized it. This actin-monomer sequestration prevents  
389 them from polymerization by changes in actin conformation [60,62]. Many factors are  
390 actually involved in the dynamics of actin in eukaryotic cells, such as e.g. the ATP  
391 concentration or the Critical Concentration (Cc) of G-actin monomers.

392

### 393 3.5. Physiological verification of differential expression analysis

394 Transcriptomics analysis is a very powerful tool to give, in a single experiment, a broad  
395 overview of the multiple processes potentially involved in a biological response. In our  
396 study, we showed the deregulation of many transcripts encoding proteins involved in  
397 immunity and in the dynamics of actin cytoskeleton, thus suggesting an important role  
398 for these biological processes during infection of *R. philippinarum* by *V. tapetis*.  
399 Therefore, we performed additional experiments in order to check the validity of some of  
400 the major interpretations raised from the differential expression analysis.

401

402 First, RNA-seq data showed a downregulation of many transcripts encoding proteins  
403 involved in the lysosomal activity, thus suggesting a crucial of related biological process  
404 during infection. We thus aimed at checking the actual effect of *V. tapetis* exposure on  
405 the lysosomal activity of *R. philippinarum* hemocytes, by using the LysoTracker assay,  
406 which is commonly recognized as an efficient proxy of the amount and activity of acidic  
407 organelles within eukaryotic cells.

408 As previously described [7,8], we first confirmed that the exposition to *V. tapetis*  
409 CECT4600 induces a loss of hemocytes adherence as compared to the control (Fig. 1),  
410 and results in a rounded phenotype. In these experimental conditions, we observed that

411 the loss of adherence is accompanied by a two-fold decrease of acidic organelles in  
412 hemocytes exposed to *V. tapetis* CECT4600 as compared to the control (Fig. 2). As a  
413 consequence, we hypothesize that the general depletion of lysosomal transcripts  
414 observed in the RNAseq experiment should result from the decreased number of  
415 lysosomes in EPFs-derived hemocyte cells.

416  
417 Second, as many transcripts involved in actin-cytoskeleton dynamics were deregulated,  
418 we aimed at observing the F-actin filaments in hemolymph that had been exposed or not  
419 to *V. tapetis* (Fig. 3), by performing a phalloidin staining experiment. Results presented  
420 in the Fig. 3 clearly show the loss of pseudopods and rounding of hemocytes after *V.*  
421 *tapetis* exposure (Fig. 3C and 3D). In these conditions, it is noteworthy that the F-actin  
422 filaments were clearly mainly located at the periphery of cells in healthy hemocytes (Fig.  
423 3B), while phalloidin staining revealed a diffuse localization of F-actin in cells exposed to  
424 *V. tapetis* (Fig. 3D). These results are consistent with previous works showing F-actin  
425 network disorganization in diseased hemocytes in another clam species, *Mya arenaria*  
426 [63], as well as with the infection model developed by Paillard [6] considering that *V.*  
427 *tapetis* induces a loss of phagosome-lysosome formation during infection of the Manila  
428 clam *R. philippinarum*.

429  
430 3.6. Hypothetical model of clam-*Vibrio tapetis* interaction  
431 We took advantage of our data to propose an hypothetical model that encompasses  
432 bacterium recognition (through microbe-associated molecular pattern [MAMP]) and  
433 some relevant metabolic processes overrepresented in the transcriptomic profile of  
434 clams EPFs in response to *V. tapetis*. Altogether, these results are consistent with the

435 knowledge we have about the influence of the BRD on actin cytoskeleton [13]. Indeed,  
436 in the case of *R. philippinarum*'s infection by *V. tapetis*, the bacterium is phagocytosed into  
437 clam's hemocytes which leads to hemocytes actin-cytoskeleton disorganization. The  
438 result of this perturbation is the loss of pseudopods (ie. rounding phenotype) and then  
439 the loss of hemocytes adhesion properties. This consequence of *V. tapetis* infection on  
440 clams is commonly used to measure strains virulence by *in vitro* characterization [7].

441 The fact that transcripts such as coactosin or profilin, that interact with either capping  
442 proteins and/or ABPs, are downregulated during infection, let us to formulate different  
443 hypothesis. Considering our results and the knowledge on actin dynamics, we made the  
444 hypothesis that infection might trigger a deregulation of capping protein which would  
445 lead hemocytes to be blocked in a "resting cell state". According to our model, capping  
446 proteins would block the + end polymerization in infected hemocytes, then increasing  
447 the levels of free G-actin [60]. In this case, Mannherz and Hannappel [60] described that  
448 in case of resting cells,  $\beta$ -thymosins massively bound to free G-actin, thus decreasing its  
449 concentrations, thus stalling net polymerization [60]. On the other hand, they also report  
450 that during this process, profilin concentration is low and most of it is inactivated. This  
451 hypothesis would need additional experiments but might explain the disorganization of  
452 the F-actin network observed during infection that gives to hemocytes this 'rounded'  
453 phenotype after challenge with *V. tapetis*.

454  
455 Figure 4A to 4C represent the conceptual model of the Manila clam EPF infection by *V.*  
456 *tapetis* in order to understand functions and interactions between the proteins that were  
457 deregulated in our experiments, and their place in the immune response to infection and

458 actin dynamics polymerization process. Figure 4A represents the immune response of  
459 healthy hemocytes to foreign bacteria. Foreign bacteria are opsonized and phagocytized  
460 by hemocyte. After fusion between the phagosome and the lysosome, bacteria are  
461 destroyed. Figure 4B represents early stage of hemocytes infection by *V. tapetis* and  
462 thus downregulation of transcripts related to pathogen recognition (C1q proteins and  
463 lectin) or to decrease of lysosomal activity as demonstrated in this study. Figure 4C  
464 represents the deregulation of the immune system in hemocytes infected by *V. tapetis*  
465 inducing a rounding phenotype related to infection with absence of pseudopods  
466 formation.

467

#### 468 4. CONCLUSION

469 This study presents the first transcriptomic approach on EPFs of experimentally infected  
470 clams. It brought novel insights on the interaction between *V. tapetis* and clam by  
471 exploring *in vivo* gene expression during the infection. This study revealed a  
472 downregulation of the immune response, especially the complement pathway and  
473 lysosomal activity, the disorganization of the actin cytoskeleton, accompanied by  
474 ribosomal genes deregulation.

475

## 476 FUNDINGS

477 This project received grants from the H2020 European project "VIVALDI" (grant  
478 agreement N°678589), CNRS INSU EC2CO-MicrobiEn "MICROPAL", CAPES-  
479 COFECUB (MICROBIV) and PVE Brésil. This work was also supported by the University  
480 of Western Brittany (UBO), and the "investment for the future" programs LabexMER  
481 (ANR-10-LABX-19) and ISblue (ANR-17-EURE-0015).

482

## 483 ACKNOWLEDGMENTS

484 We warmly thank Fabien Fonteneau (EARL Les Claires de Bonsonge, Marennes,  
485 France) for providing the clams. We thank colleagues from the Federal University of Rio  
486 De Janeiro, Brazil who welcomed us in Rio. We thank N. Le Goïc, F. Nunez, H. Hégaret,  
487 C. Fabioux, I. Paul-Pont, F. Le Grand, F. Riera, AL. Cassone, P. Le Souchu, A. Muir, E.  
488 Harney, Y. Epelboin, M. Fuhrmann, M. Provost, L. Frère, F. Boullot, B. Dubief, M.  
489 Czamanski, R. Morvezen, V. Foulon, M. Protat, C. Quéré, and A. Huvet for their help for  
490 animals conditioning, injections and samplings.

491

## 492 AUTHOR CONTRIBUTIONS

493 CP, FT and VP acquired funds and coordinated the study. GR, AB and CP designed  
494 and performed the experimental infection. AB performed RNA extractions and libraries  
495 preparations, with the advices of LO. LO and FT performed DNA sequencing. AR and  
496 EC performed bioinformatics analysis, with inputs from VP. AR and CL performed the  
497 flow cytometry analyses. AR, VP and CP wrote the article (the original draft was written  
498 by AR). The article was carefully reviewed by other co-authors, who all approved the  
499 final version.

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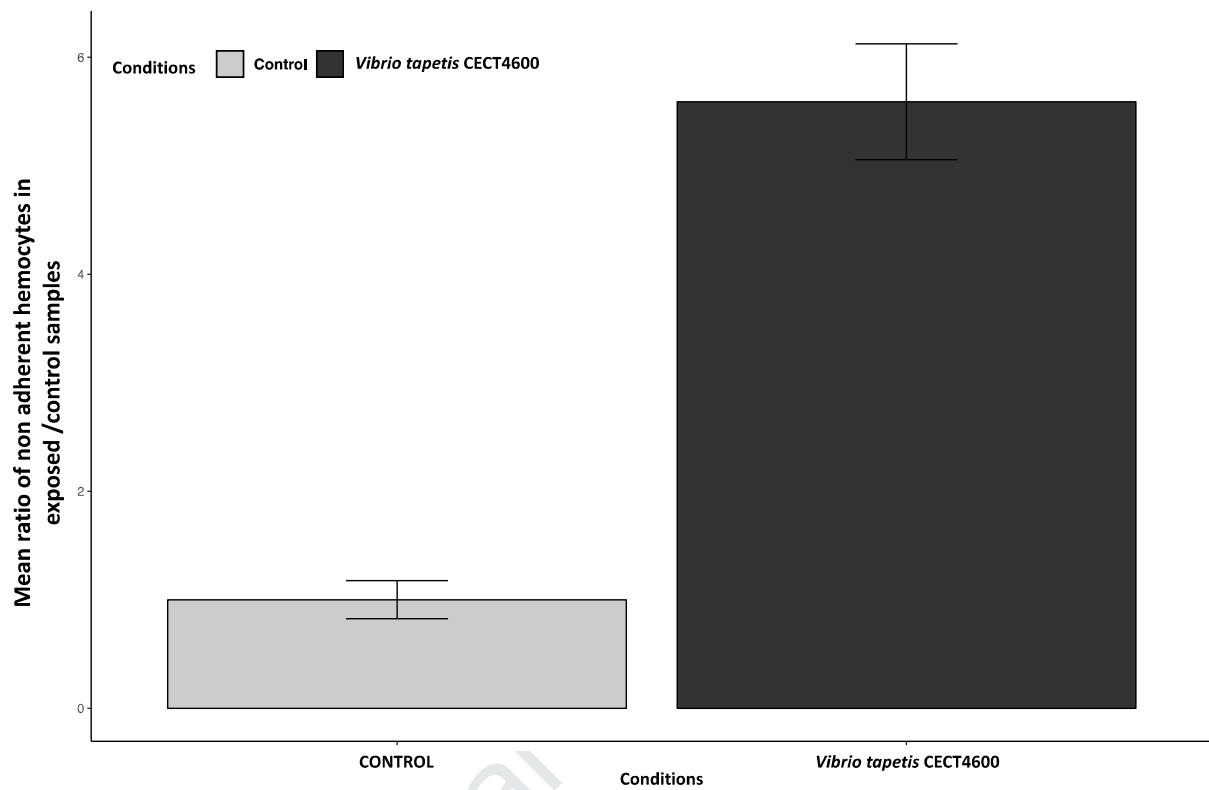
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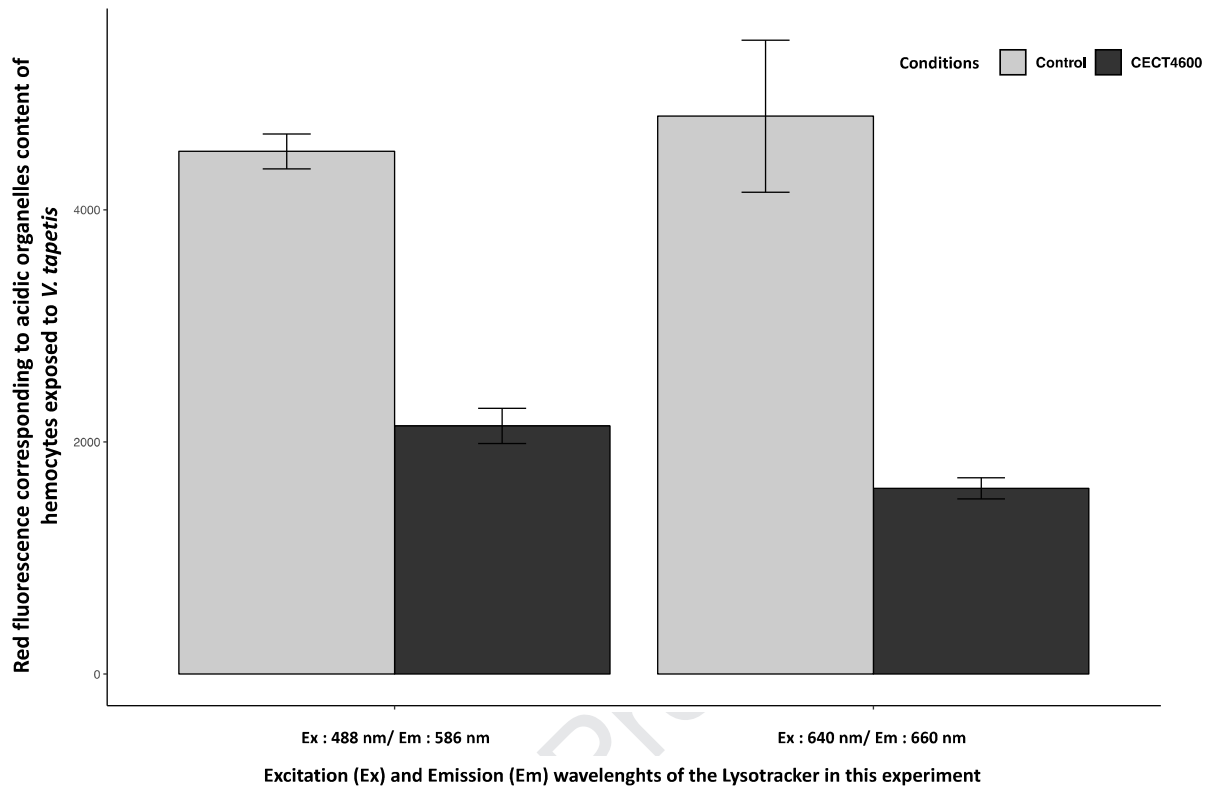


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688 Figure 1: Adherence test on hemocytes exposed to *Vibrio tapetis* CECT4600 (black) or  
689 non-exposed (grey) representing the mean ratio of non-adherent hemocytes on exposed  
690 / control samples. Error bars = Standard error.

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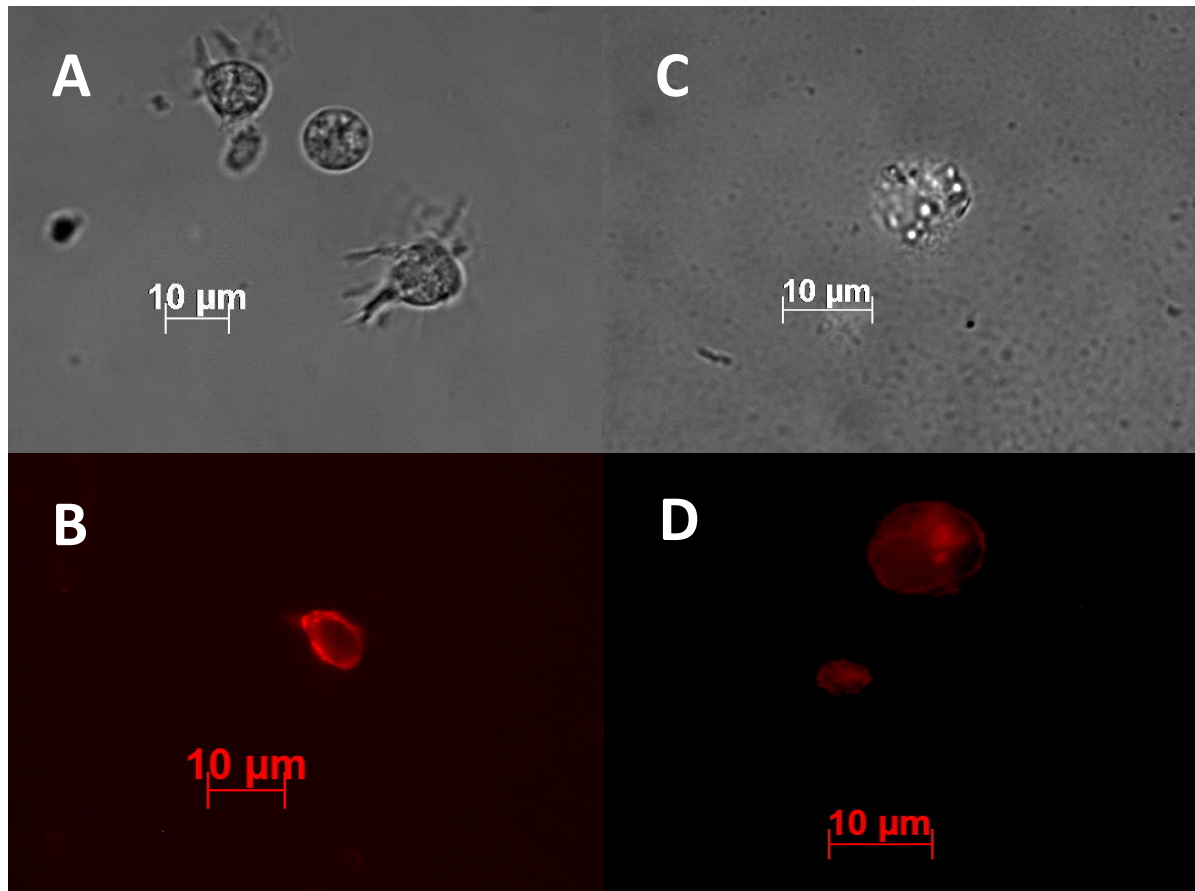


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694 Figure 2: Mean red fluorecence of Lysotracker red used as a measure of the amount of  
695 acidic organelles in hemocytes, exposed to *V. tapetis* CECT4600 (black) or non-  
696 exposed (grey). Error bars = Standard error.

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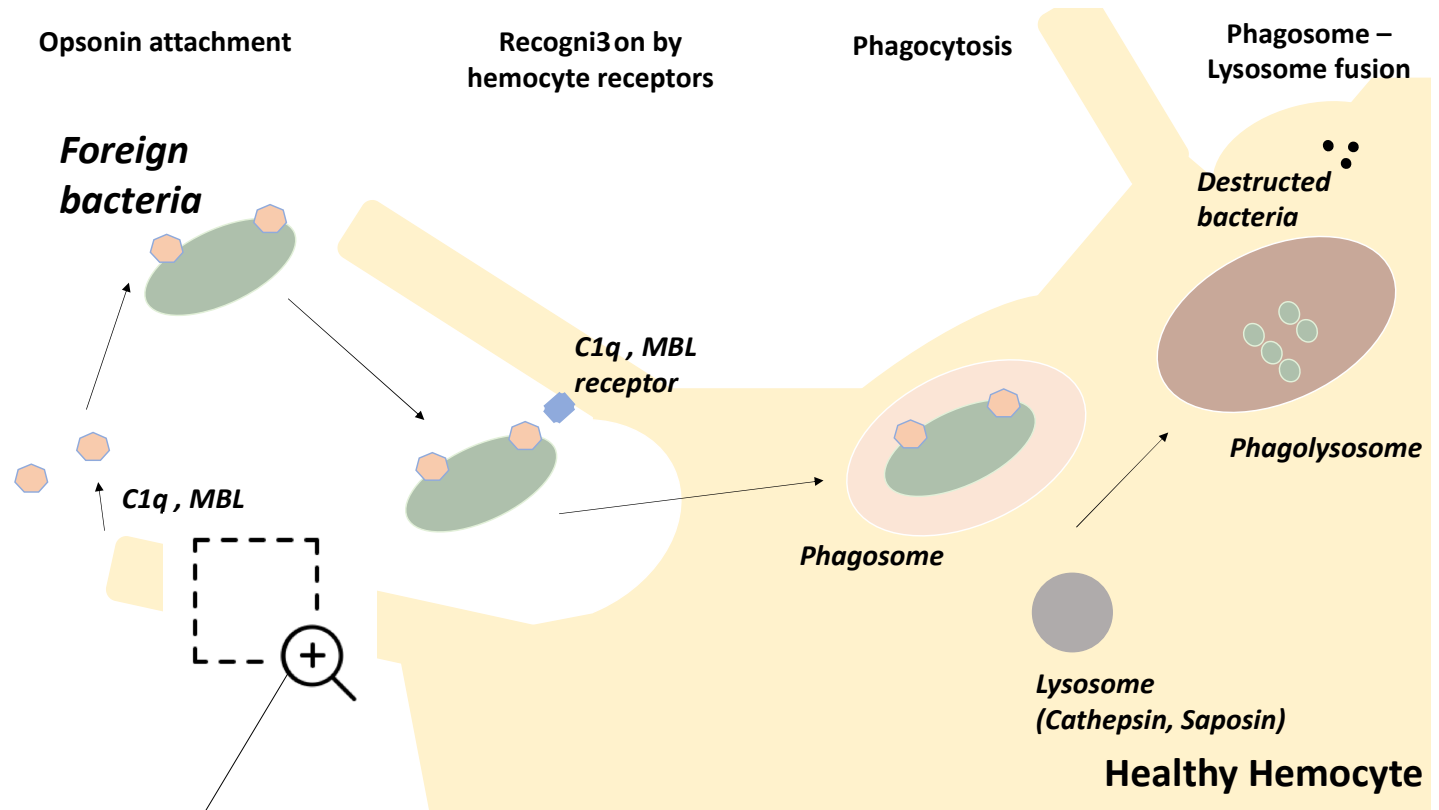


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701 Figure 3: Rhodamine phalloidin staining of F-actin filaments in hemocytes that had been  
702 exposed (C, D) or not (A, B) to *V. tapetis* CECT4600. A, C: bright field microscopy  
703 observations of control (A) and exposed (C) hemocytes. B, D: epifluorescence  
704 observations of control (B) and exposed (D) hemocytes. Wavelengths: excitation, 550  
705 nm; emission, 605 nm. The scale bars (2 cm=10 μm) correspond to the pictures A, B, C  
706 & D.

707 **Figure 4: The hypothetical model of clam – *V. tapetis* interaction**



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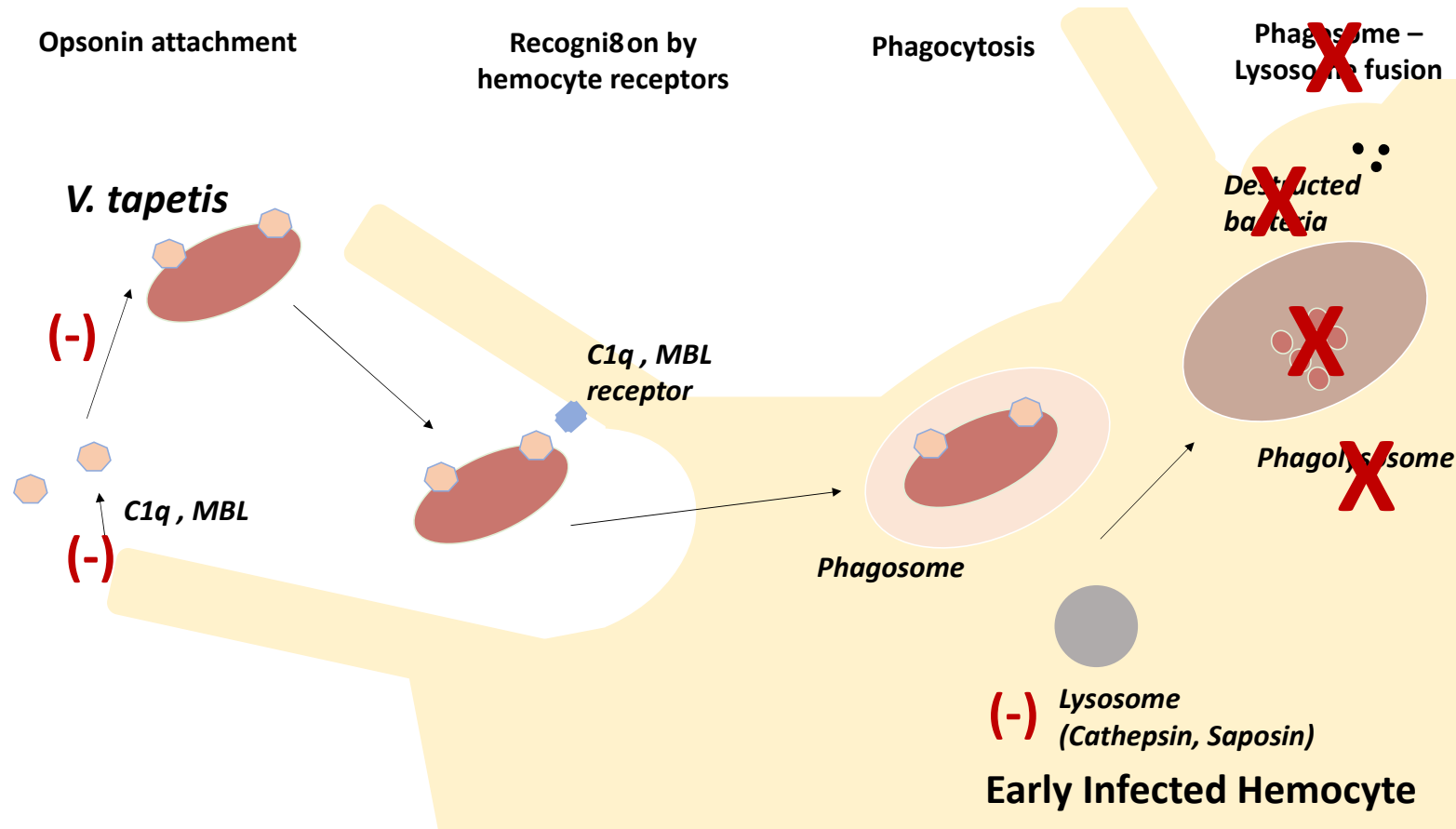


Figure 4B: Immune response of clams to *V. tapetis* on the first steps of infection in early Infected Hemocytes. (-) represent down regulated genes in this study

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767 Figure 4C: Immune response of clams to *V. tapetis* on the  
768 late steps of infection in infected, non-functional and,  
769 according to our model, resting hemocytes. (-) represent  
770 down regulated genes in this study.

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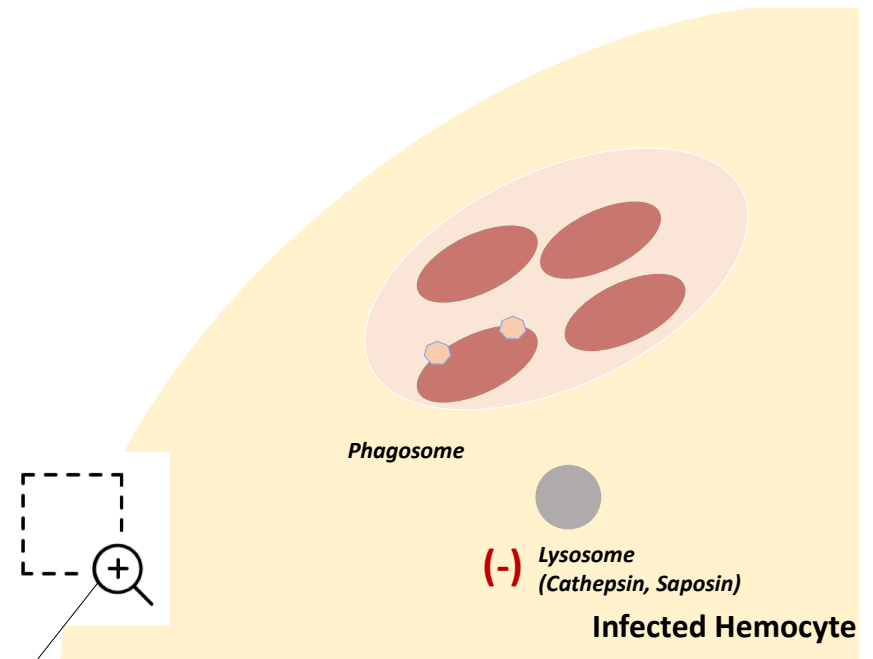
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**No pseudopods formation : disorganization of F-actin  
after challenge with *V. tapetis***

## HIGHLIGHTS

- Brown Ring Disease (BRD) is due to the bacterium *Vibrio tapetis*, and affects the Manila clam *Ruditapes philippinarum*
- This study is the first transcriptomic approach based on collection of extrapallial fluids (EPFs) on living animals experimentally infected by *V. tapetis*.
- Transcripts differentially expressed in infected clams are mainly involved in the immune system, translation and in actin cytoskeleton organization
- We showed a downregulation of the immune system related to the complement pathway through C1q and lectin during infection
- We highlighted a deregulation of transcripts involved in actin organization related to capping proteins
- Physiological validations of immune and actin deregulation were performed
- This study opens new perspectives to explore pathogenicity in the case of BRD.