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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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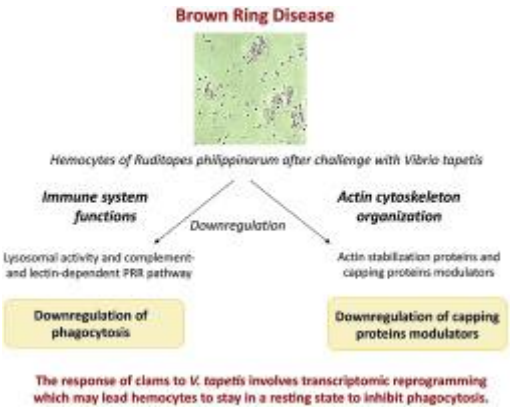
Abstract :

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 β12-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

Abbreviations

ABP: Actin Binding Protein

BRD: Brown Ring Disease

EPF: Extrapallial Fluids

FSW: Filter Sterilized Seawater

1. INTRODUCTION

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

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

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

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

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

2. MATERIALS AND METHODS

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

Animals and experimental design are fully described in [14]. Two years-old clams were harvested in the Marennes-Oleron Bay (Charente Maritime, France) on May 5th, 2014. A first visual health diagnostic was performed on 50 clams *in situ* and no BRD clinical signs were observed. Clams were then transferred to Ifremer facilities in Plouzané (Finistère, France) and held in a 6-day quarantine with a chloramphenicol (8 $mg.L^{-1}$) treatment to avoid local contamination. After quarantine, animals were randomly divided and maintained in trays placed in eighteen 100 L-tanks equipped with air-lift systems (100 clams per tank). Water temperature was maintained at 13°C during the whole experiment so that infection by *V. tapetis* was favored [5]. Tanks were continuously supplied with filter sterilized seawater (FSW, filtered at 1 µm and UV-sterilized) at a renewal rate of 50% per day and cleaned every two days. Clams were fed *ad libitum* with T-iso. Manila clams, *R. philippinarum*, were injected in the extrapallial cavity with 100 µL of *V. tapetis* CECT4600 suspension at the concentration of 10^7 $CFU.mL^{-1}$

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

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

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

2.3. Clams *in vitro* infection : bacterial exposure

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

2.3.1. F-actin visualization by microscopy

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

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

2.3.2. Lysotracker assay

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

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

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

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

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

3. RESULTS AND DISCUSSION

3.1. Transcriptomic results: differential expression analysis

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

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

233 Table 1: Number and functions of the transcripts differentially expressed in clams EPFs during *V. tapetis*
234 infection
235

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

236
237

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

DOWN REGULATED IN INFECTED CLAMS			UP REGULATED IN INFECTED CLAMS		
Seq. Description	logFC	FDR	Seq. Description	logFC	FDR
Immune system			**Immune system**		
cathepsin K-like	-10.52	1.25E-05	*complement C1q-like protein 4*	11.02	4.29E-04
cathepsin K	-10.04	4.87E-04	cyclophilin-like protein	9.45	3.93E-04
cathepsin l	-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 l	-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
saposin B domain-containing protein precursor	-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			
complement C1q-like protein 4	-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			
Actin cytoskeleton organization			**Actin cytoskeleton organization**		
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	*Kinesin*	10.33	4.34E-08
Myophilin	-10.12	1.98E-05	*actin*	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			

3.2. Immune response

Extrapallial fluids (EPFs) play an essential role in the biomineralization of the shell of bivalves. Given the particular etiology and symptoms of BRD, this compartment, containing numerous hemocytes [24], is particularly interesting to better understand both the infection process and how the clam's hemocytes fight against it. For this reason, in this study, *V. tapetis* infection was carried out directly inside the EPFs passing through the periostracal lamina barrier to accelerate the immune response, particularly in EPFs.

A transcript encoding lysozyme was found to be upregulated in response to *V. tapetis* infection. In previous studies, lysozyme activities in cell lysates of extrapallial fluids were significantly higher 7 days after *V. tapetis* inoculation into the pallial cavity, as compared to control ones [24]. The antibacterial protein lysozyme, which is circulating in body fluids such as hemolymph and EPFs, is an important line of defense of the animal against bacterial pathogens because of their bacteriolytic properties [5,25].

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

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

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

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

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

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

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

3.3. Ribosomal proteins

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

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

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

3.4. Actin cytoskeleton organization

Actin filaments (F-actin) belong to the cell cytoskeleton and are dynamically polymerized and depolymerized from monomers of actin (G-actin) into the cell to enable cellular activities such as locomotion, exocytosis or phagocytosis. The actin cytoskeleton is also responsible for the cell shape. In this study, we showed that at least 27 transcripts involved in actin cytoskeleton organization were differentially expressed during infection, among which 9 were induced while 18 had their expression reduced. Genes downregulated during infection are mainly involved in actin filament stabilization such as myophilin, a member of calponin protein family [50,51], cysteine glycine rich protein (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 β4-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 β4-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 β12 thymosin transcripts during
385 infection by *V. tapetis*. β12-thymosin belongs to the ABPs family. Fifteen variants of β-
386 thymosin proteins have been described in many vertebrates and invertebrates, but not

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

3.5. Physiological verification of differential expression analysis

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

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

As previously described [7,8], we first confirmed that the exposition to *V. tapetis* CECT4600 induces a loss of hemocytes adherence as compared to the control (Fig. 1), 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

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

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

Figure 4A to 4C represent the conceptual model of the Manila clam EPF infection by *V. tapetis* in order to understand functions and interactions between the proteins that were 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

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AUTHOR CONTRIBUTIONS

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

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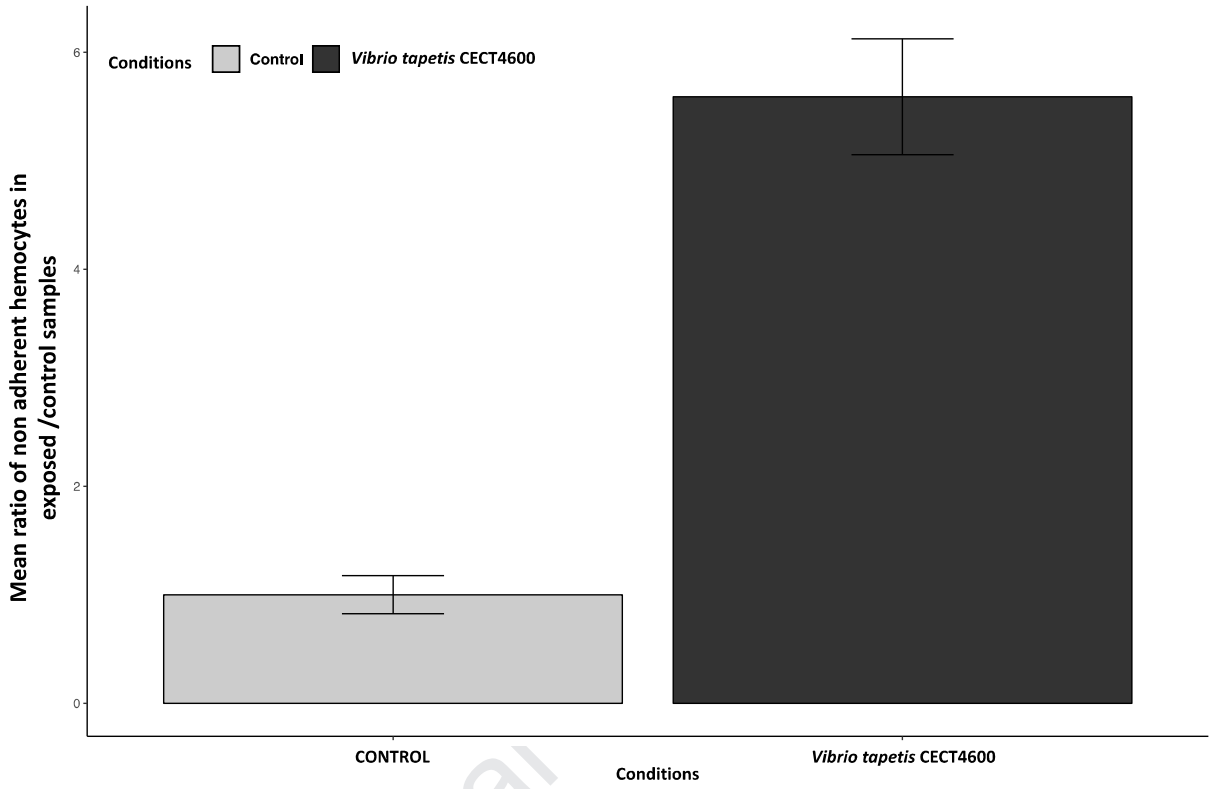

687

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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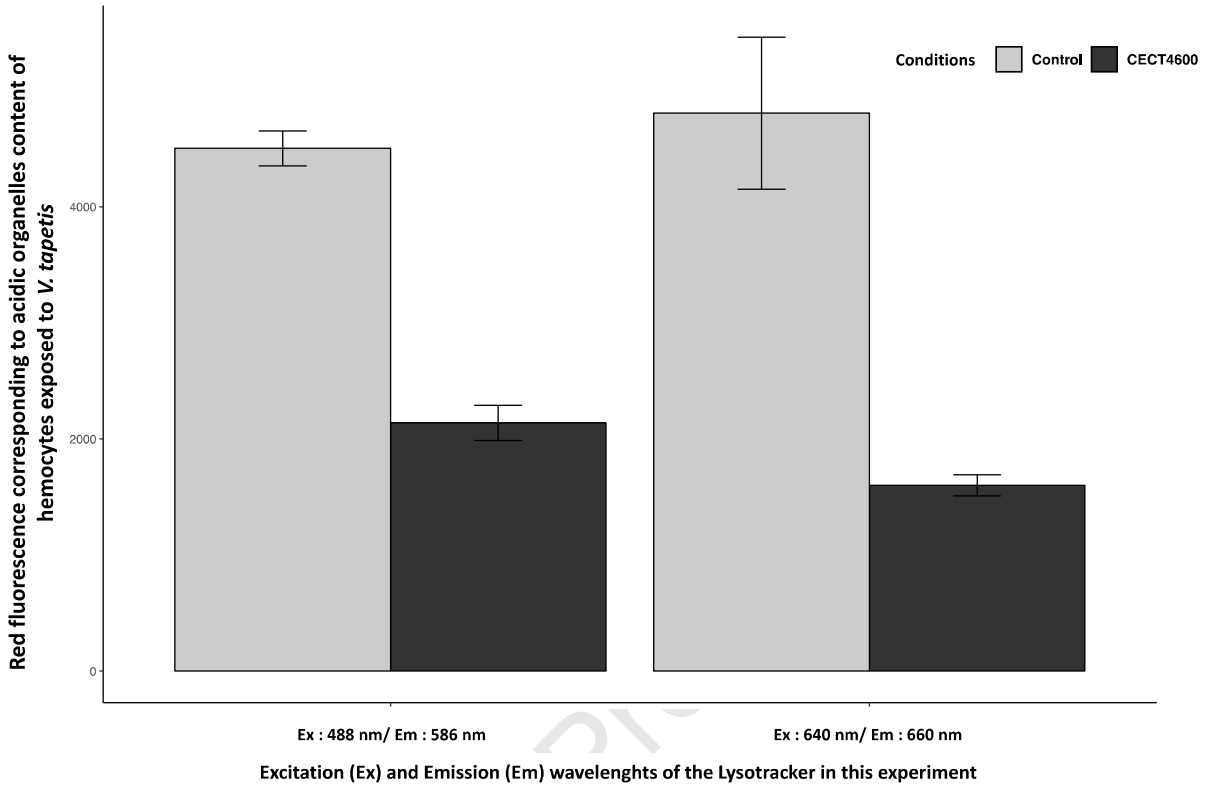

693

694 Figure 2: Mean red fluorescence 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.

697

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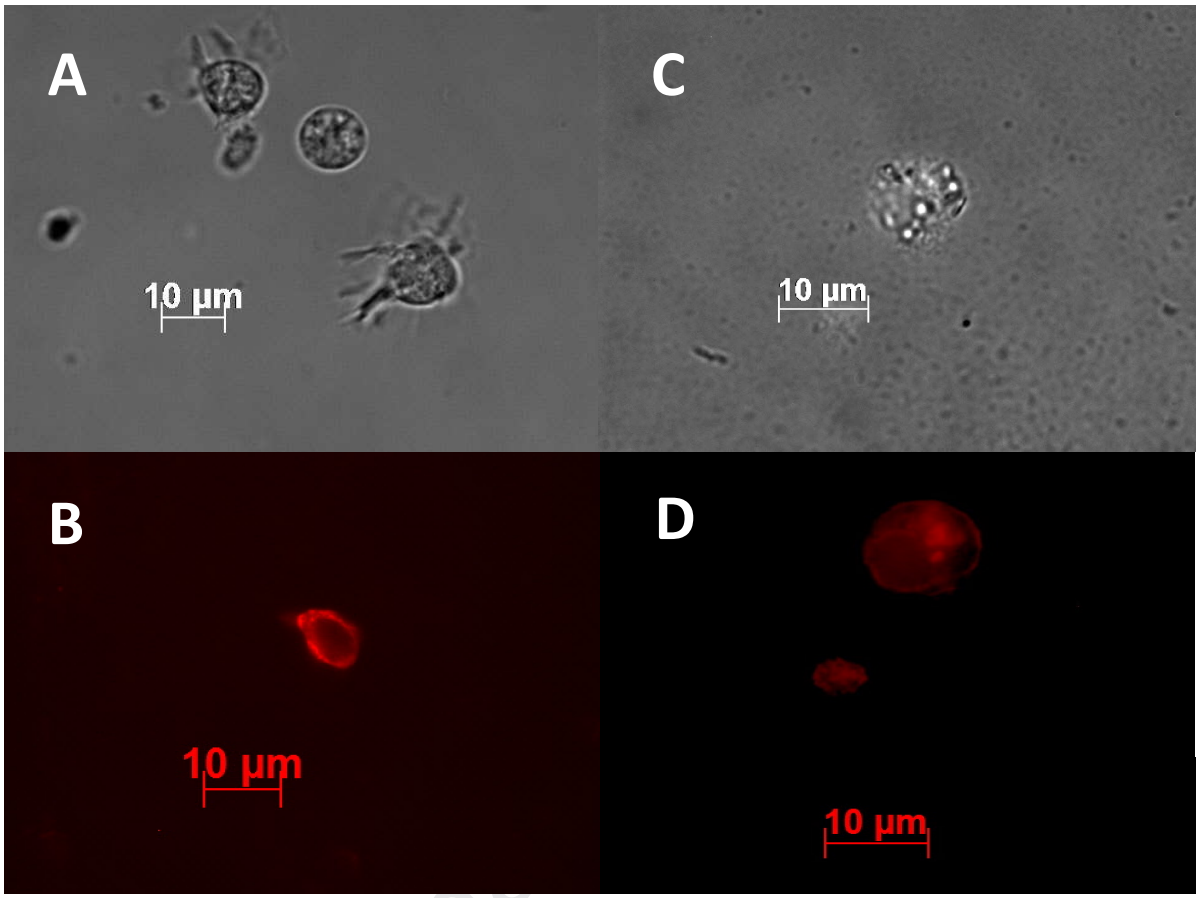


Figure 3: Rhodamine phalloidin staining of F-actin filaments in hemocytes that had been exposed (C, D) or not (A, B) to *V. tapetis* CECT4600. A, C: bright field microscopy observations of control (A) and exposed (C) hemocytes. B, D: epifluorescence observations of control (B) and exposed (D) hemocytes.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

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

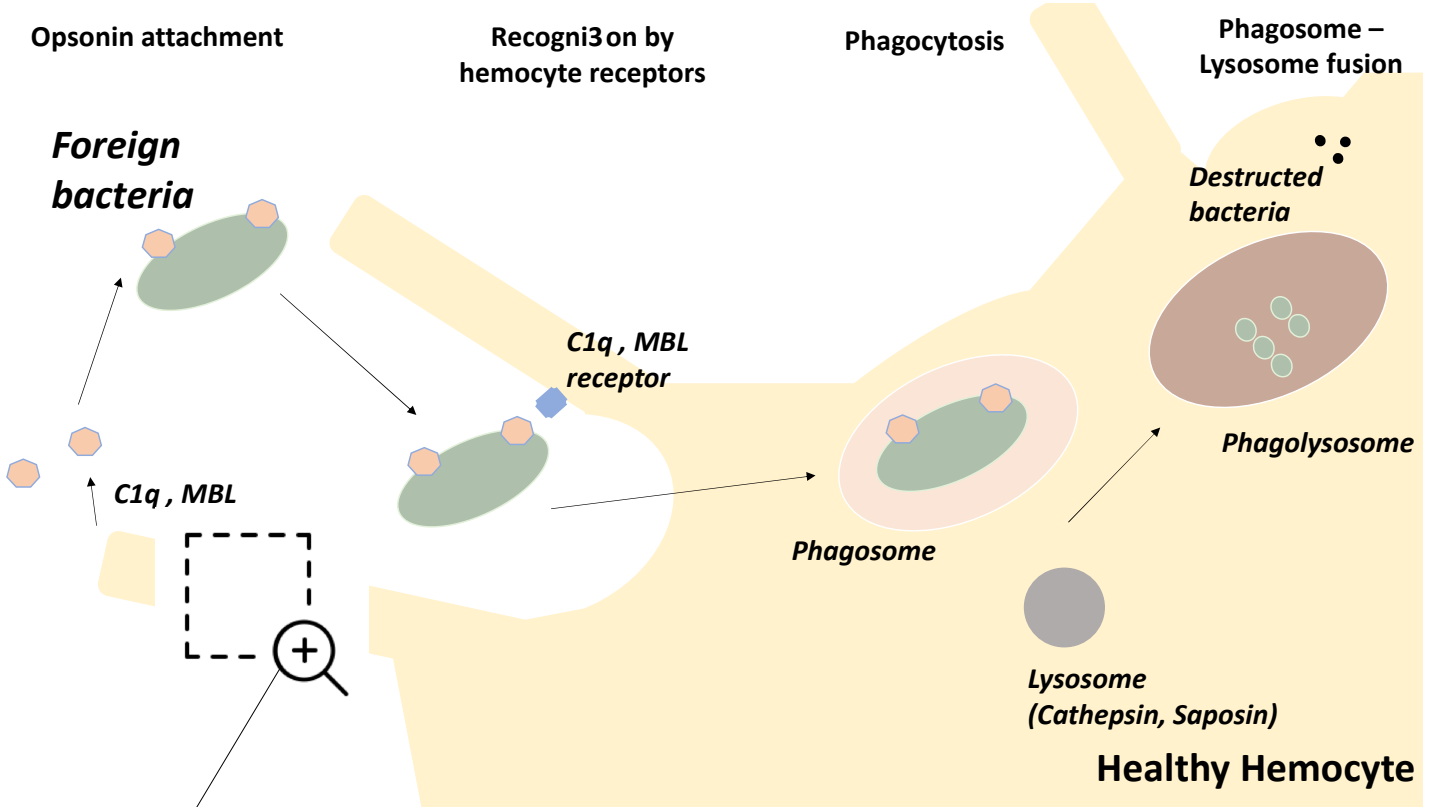


Figure 4A: Immune response of clams to foreign bacteria in healthy, functional and active hemocytes

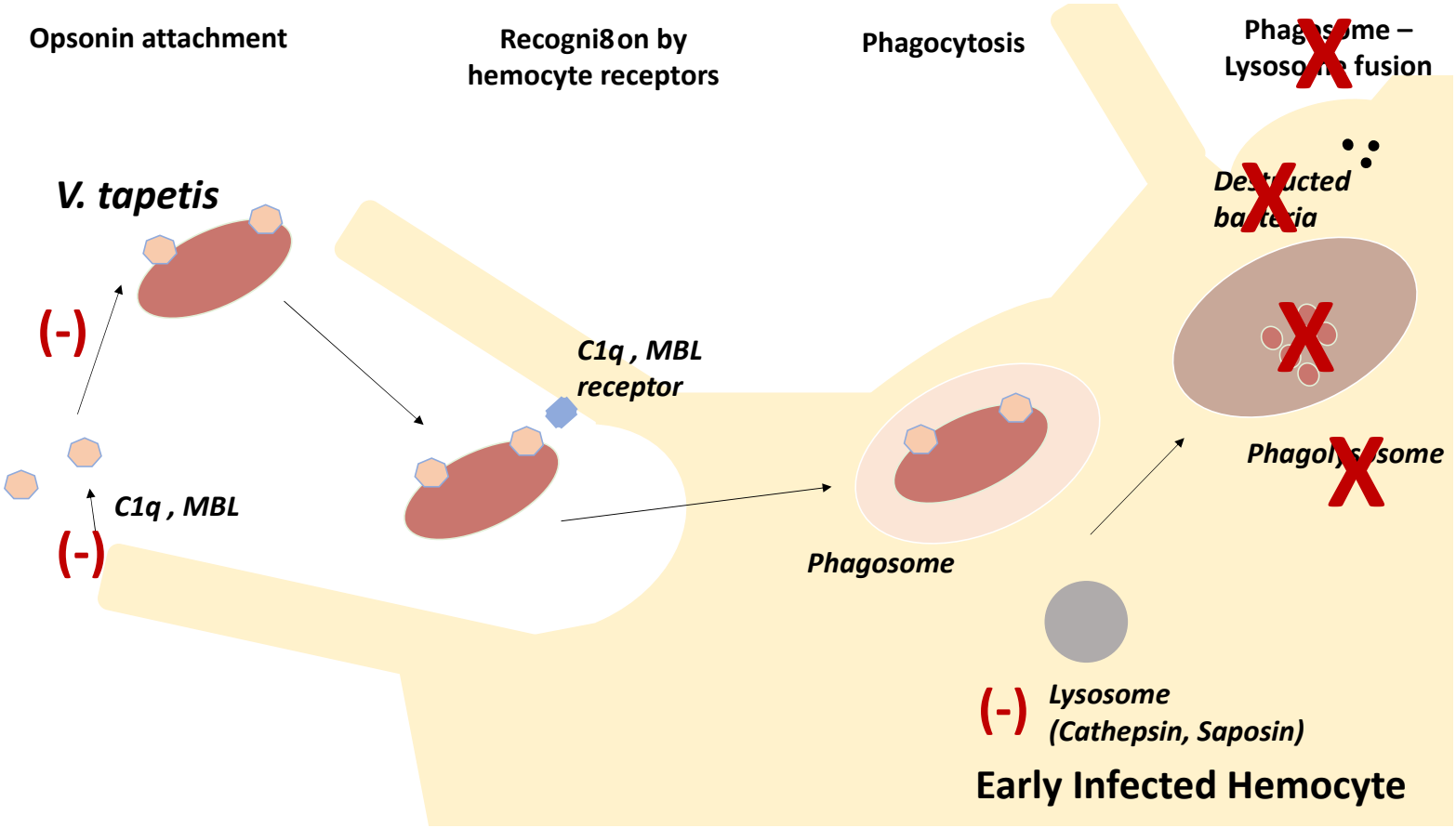
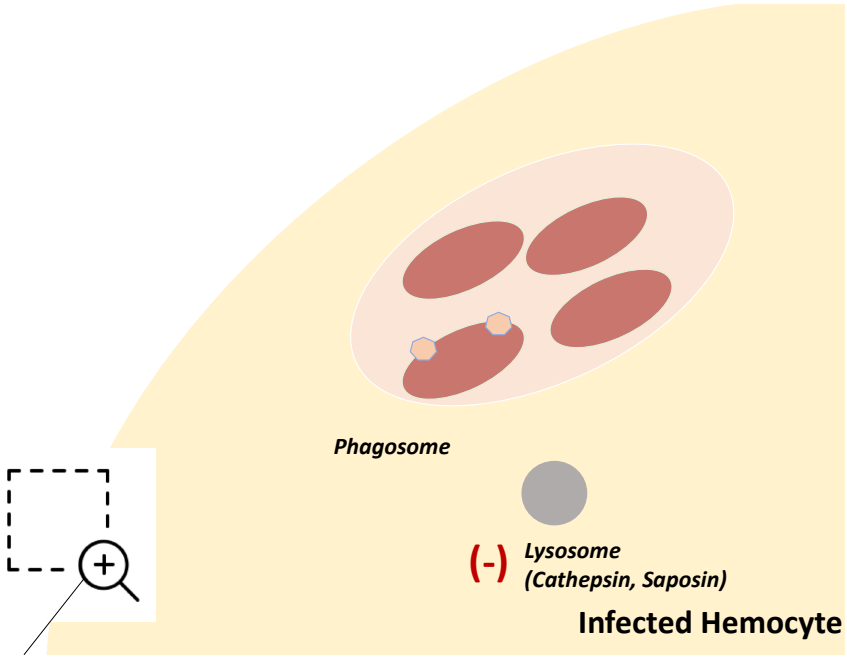


Figure 4B: Immune response of clams to *V. tapetis* on the first steps of infection in early Infected Hemocytes. **(-)** represent down regulated genes in this study

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



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.