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Transcriptional changes in Manila clam (*Ruditapes philippinarum*) in response to Brown Ring Disease

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ABSTRACT

Brown Ring Disease (BRD) is a bacterial infection affecting the economically-important clam *Ruditapes philippinarum*. The disease is caused by a bacterium, *Vibrio tapetis*, that colonizes the edge of the mantle, altering the biomineralization process and normal shell growth. Altered organic shell matrices accumulate on the inner face of the shell leading to the formation of the typical brown ring in the extrapallial space (between the mantle and the shell). Even though structural and functional changes have been described in solid (mantle) and fluid (hemolymph and extrapallial fluids) tissues from infected clams, the underlying molecular alterations and responses remain largely unknown. This study was designed to gather information on clam molecular responses to the disease and to compare focal responses at the site of the infection (mantle and extrapallial fluid) with systemic (hemolymph) responses. To do so, we designed and produced a Manila clam expression oligoarray (15K Agilent) using transcriptomic data available in public databases and used this platform to comparatively assess transcriptomic changes in mantle, hemolymph and extrapallial fluid of infected clams. Results showed significant regulation in diseased clams of molecules involved in pathogen recognition (e.g. lectins, C1q domain-containing proteins) and killing (defensin), apoptosis regulation (death-associated protein, bcl-2) and in biomineralization (shell matrix proteins, perlucin, galaxin, chitin- and calcium-binding proteins). While most changes in response to the disease were tissue-specific, systemic alterations included co-regulation in all 3 tested tissues of molecules involved in microbe recognition and killing (complement-related factors, defensin). These results provide a first glance at molecular alterations and responses caused by BRD and identify targets for future functional investigations.

1. Introduction

Manila clam, *Ruditapes philippinarum*, is the second most economically-important bivalve species in the world after the Pacific oyster with a worldwide production exceeding 3.7 Million tons (FAO, 2014, http://www.globefish.org/homepage.html). The species was introduced for aquaculture purpose to various locations around the globe with a particular success in western Europe where it became increasingly widespread up until the late 1980's when mass mortalities in clam beds have occurred associated with Brown Ring Disease (BRD), a bacterial disease caused by *Vibrio tapetis* [1,2]. *V. tapetis* colonizes the surface of the epithelia of the mantle and the periostracal lamina causing a disruption of the normal calcification process [3,4]. This alteration provokes the progressive deposition and accumulation of the periostracal lamina on the inner surface of the shell, thereby producing the typical brown conchiolin deposit within the extrapallial space [3–5]. Although the pathogen usually remains confined to the periostracal lamina, the organic deposit and the extrapallial fluid of clams with mild to moderate infections, *V. tapetis* has been observed in mantle tissue and within digestive cells of severely infected individuals [6,7]. Evidence suggests that the bacteria can penetrate into the mantle through lesions in the epithelium of heavily infected clams, subsequently spreading throughout the body to cause severe systemic infection and death [8].

Like all invertebrates, *R. philippinarum* relies on its innate immune system to fend invaders. Even though evidence shows some

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levels of specificity of the innate immune system [9–11], it is largely considered as a rather "generic" immune system mediated by cellular (hemocytes) and humoral (macromolecules) components present throughout the body cavity that provide an immediate way to fight pathogens [12]. Hemocytes use phagocytosis or encapsulation to eliminate undesirable particles (e.g. pathogens, abnormal/dead cells) and contribute to the secretion of humoral factors. Multiple circulating bioactive molecules have been found to directly or indirectly contribute to the elimination of invaders. For example, lectins are known to be involved in non-self recognition, phagocytosis and encapsulation [13,14]. Antimicrobial peptides (among which defensins) and lysozymes contribute to the direct killing of pathogens [15,16]. These antimicrobial actions are orchestrated by a wide variety of signaling and feedback pathways that regulate the innate immune system in invertebrates, involving multiple signaling intermediates such as kinases and phosphatases [12].

For nearly 25 years, several studies have focused on the investigations of *R. philippinarum-V. tapetis* interactions during the development of BRD. Most of these studies targeted cellular and biochemical changes in clams in response to the infection [17–24]. Results highlighted the importance in BRD of defense factors (hemocytes, diverse bioactive compounds) associated with the extrapallial fluid which represents the first immunological barrier against *V. tapetis* [21–23,25]. Recently, small scale investigations of molecular interactions between clams and *V. tapetis* were carried out using subtractive suppressive hybridization (SSH) libraries of hemocytes exposed to the pathogen in vitro [26]. Results showed significant regulation in hemocytes of challenged *R. philippinarum* of genes involved in cytoskeleton organization [26], supporting previous cellular bioassay results showing a potent cytotoxic effect of *V. tapetis* to clam hemocytes [8,27–29]. Similarly, SSH results on mantle tissues from experimentally-infected clams showed a significant regulation of genes involved in biomineralization and antimicrobial defense, such as carbonic anhydrase, a big defensin and a serine protease inhibitor (serpin) [30].

Recent development in sequencing technologies provided new tools for the investigation of molecular host–pathogen interactions in non-model species. High throughput gene expression techniques such as DNA microarray analyses led to the emergence of a new field in immunology called immunogenomics which is defined as the study of gene and protein expression related to the immune system [31,32]. Genomics has significantly enhanced our knowledge of immunity by improving our understanding of regulatory pathways during host–pathogen interactions, and developing a physiological perspective on the environmental facilitation of infection. For instance, transcriptome profiling can be used to provide "physiological fingerprints" of healthy versus diseased (or susceptible versus resistant) organisms at the level of gene expression. The availability of transcriptomic data increased exponentially for *R. philippinarum* with the publication in 2011 of the first transcriptome for the species generated using high-throughput sequencing (Roche 454 technology) of a normalized cDNA library [33], followed shortly with a characterization of the transcriptome of hemocytes exposed in vitro to several immune stimulants [34]. This new information was then used to devise oligoarray platforms to investigate clam response to environmental stress [35,36], or in response to in vitro exposure of hemocytes to bacterial challenge [37]. To our knowledge, there have been no previous studies employing high throughput transcriptomic techniques to investigate Manila clam response to pathogen exposure in vivo.

In the current study, we used publically-available genomic resources for *R. philippinarum* to build an oligoarray platform and employed this device to profile transcriptomic changes associated with BRD in clams. Particular focus was given to evaluate changes in the mantle (main target tissue for the infection) and to compare transcriptomic profiles in hemocytes collected from the hemolymph (systemic response to the infection) or the extrapallial fluid (focal response).

2. Materials and methods

2.1. Biological samples

Adult clams (44 ± 3 mm in length, mean ± standard deviation) were collected from an aquacultured plot located at the Ile Tudy Bay (Finistère, France) in July 2011 (n = 166). Hemolymph and extrapallial fluid were sampled individually as previously described [25,27]. Briefly, hemolymph was withdrawn from the posterior adductor muscle, through the hinge, using a sterile needle (25-gauge) fitted onto 1-mL syringe. After hemolymph collection, the clams were carefully shucked and the extrapallial fluid was collected from each valve by inserting a sterile needle between the mantle and the shell in the sinusal and central peripheral compartments [25]. Our previous studies have shown that these extrapallial compartments contain hemocytes at concentrations similar to those measured in hemolymph [21–23,25]. Hemolymph and extrapallial fluid samples were then centrifuged (785 g, 4 °C for 10 min) to separate hemocytes from the acellular fraction. Pelleted hemocytes were then immediately frozen in liquid nitrogen. In parallel, mantle tissues were dissected and immediately frozen in liquid nitrogen. Mantle and hemocyte samples were stored at −80 °C until processed for RNA extraction. Following collection, empty shells were submitted to BRD diagnostics as previously described [27]. The current study used similar-sized clams that were classified as either diseased (clams showing typical brown deposit) or healthy (no visible macroscopic or microscopic symptoms of the disease [27]). Trizol reagent (MRC, Inc., Cincinnati, OH, USA) was used to isolate RNA from preserved samples following manufacturer's protocol. RNA quality and quantity were estimated using a Nandrop spectrophotometer. Samples were pooled (same quantity of RNA from each sample) to generate 6 pools for each condition (healthy or diseased) and tissue type (extrapallial fluid, hemolymph or mantle; 3 to 4 clams/pool) for a total of 36 pools.

2.2. Oligoarray construction, hybridization and data analysis

cDNA sequence data were obtained from different sources publically available in April 2011. These included 5656 sequences obtained from the National Center for Biotechnology Information (NCBI), 32,606 sequences obtained from Ruphibase (the *R. philippinarum* transcriptome database at the University of Padova, Italy), as well as 542 sequences from subtractive suppressive hybridization experiments previously generated by our group at the European Institute for Marine Studies (IUEM, [26,30]). Sequences were annotated using the Blast2GO software system [[38], http://www.blast2go.com/b2ghome] against NCBI non redundant database (blastx, E-value cut off of 10 E^{-3}). Sequences were then cured to retain 7982 annotated sequences (1544 originating from NCBI, 6243 from Ruphibase and 195 from IUEM). These were complemented with 7102 non-annotated sequences (sequence length > 627 bp) to emphasize gene discovery (1450 from NCBI, 5620 from Ruphibase and 31 from IUEM). All sequences were submitted to Agilent eArray application (https://earray.chem.agilent.com/earray/) for probe production. Probes were synthesized in situ along with positive and negative controls using 8×15K-feature Agilent format slides (1 probe/sequence). The complete dataset can be found at the Gene Expression Omnibus public database (GEO) (http://www.ncbi.

nlm.nih.gov/geo/) under the accession number GSE58198. Labeled (Cy3 or Cy5) complementary RNA (cRNA) was synthesized from 150 ng of RNA purified from different tissues of healthy and diseased clams using the Two-Color Microarray-Based Gene Expression Analysis Protocol (Quick Amp Labeling) following manufacturer's protocol. Labeled cRNA was purified using Illustra CyScribe GFX Purification Kit (GE Healthcare). cRNA quantity and quality (including dye incorporation) were determined by spectrophotometry (Nanodrop). Samples were considered satisfactory if cRNA concentration and incorporation efficiency exceeded 300 ng/ul and 8 pmol Cy/ug cRNA, respectively. All arrays were hybridized following a balanced block design with the same amount of cRNA (300 ng of each Cy3- and Cy5-labeled cRNA). Arrays hybridization and washes were conducted according to the kit protocol and the arrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) using the suggested Agilent scan settings. Generated fluorescence intensities were normalized to remove within-array and between-array variations using the LIMMA package in R software [39]. After normalization, probes with intensities less than two-fold background intensities (in all biological replicates) were eliminated from further analysis. Data were then submitted to statistical analysis for microarray (SAM, false discovery rate set at 0.01) using the Multi Experiment Viewer (MeV) program [40]. Differences in expression between healthy and diseased clams were considered significant for genes that fulfill the following 2 criteria: (1) a one and half fold increase from the mean (up-regulation) or a one and half fold decrease from the mean (down-regulation), and (2) shown to be statistically significant in SAM. Significantly regulated transcripts were re-blasted in fall 2013 using Blast2GO to update the annotations. Blast2GO was also used for performing enrichment analyses by means of Fisher's exact test with multiple testing correction (Benjamini and Hochberg) of false discovery rate ($p < 0.05$).

3. Results

3.1. Overall gene regulation in diseased clams

The number of spots that produced a fluorescent signal 2-fold higher than background was 9880 (65%) for the extrapallial fluid 10,575 (70%) for the hemolymph and 6037 (40%) for the mantle. Among these, a total of 1059 were significantly up-regulated and 641 were down-regulated across all tissues combined (Supplementary Tables S1 to S6). In the extrapallial fluid, a total of 683 transcripts were up-regulated (393 annotated and 290 non-annotated) and 296 transcripts were down-regulated (194 annotated and 102 non-annotated) (Supplementary Tables S1 and S2). In the hemolymph, 503 transcripts were up-regulated (250 annotated and 253 non-annotated) and 330 transcripts were down-regulated (196 annotated and 134 non-annotated) (Supplementary Tables S3 and S4). The number of up-regulated transcripts in the mantle was 146 (65 annotated and 81 non-annotated) while 132 transcripts (62 annotated and 60 non-annotated) were down-regulated (Supplementary Tables S5 and S6). The distributions of Gene Ontology (GO) terms were similar between up- and down-regulated transcripts when considering all tissues combined (Supplementary Figure S1) but trends become more obvious when data is analyzed on a tissue-by-tissue basis (Figs. 1 and 2). This is particularly true in the extrapallial fluid where the biological process GO term "immune system process" (GO:0002376) was represented by 1 down-regulated and 8 up-regulated transcripts. The term "response to stimuli" (GO:0050896) followed the same trend but to a lesser extent with 10 down-regulated and 17 up-regulated transcripts (Fig. 1). The distribution of GO terms in the hemolymph also showed a higher representation of the GO term: "immune system process" among up-regulated transcripts (7 occurrences) as compared to down-regulated transcripts (2 occurrences) (Fig. 1).

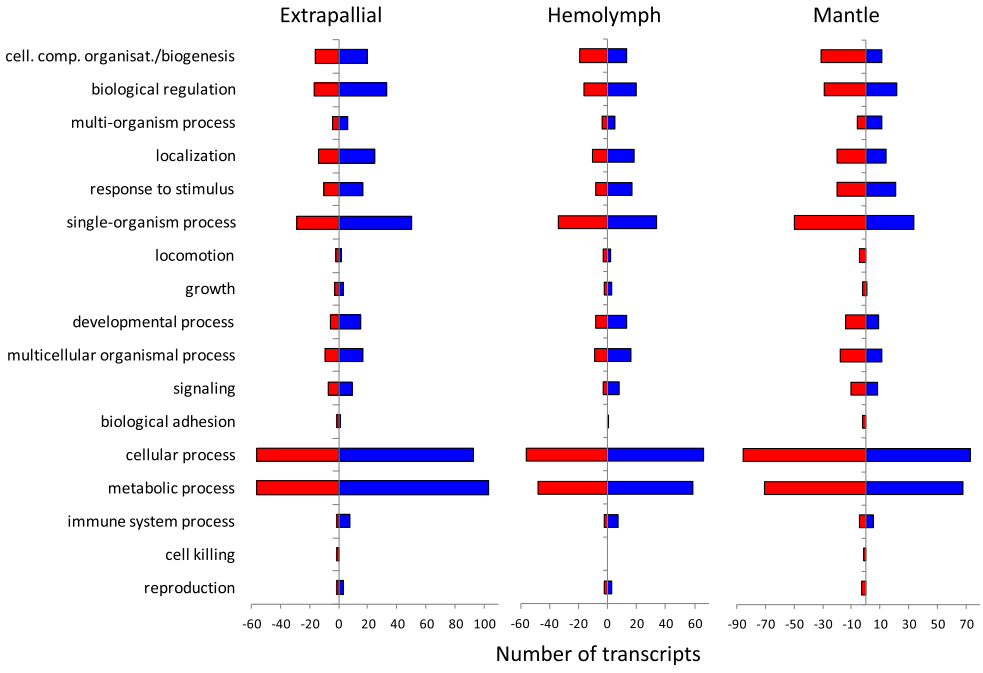

Fig. 1. Biological processes (GO terms, level 2) regulated in different tissues from infected clams as compared to healthy individuals. The number of down-regulated (negative) and up-regulated (positive) transcripts is given.

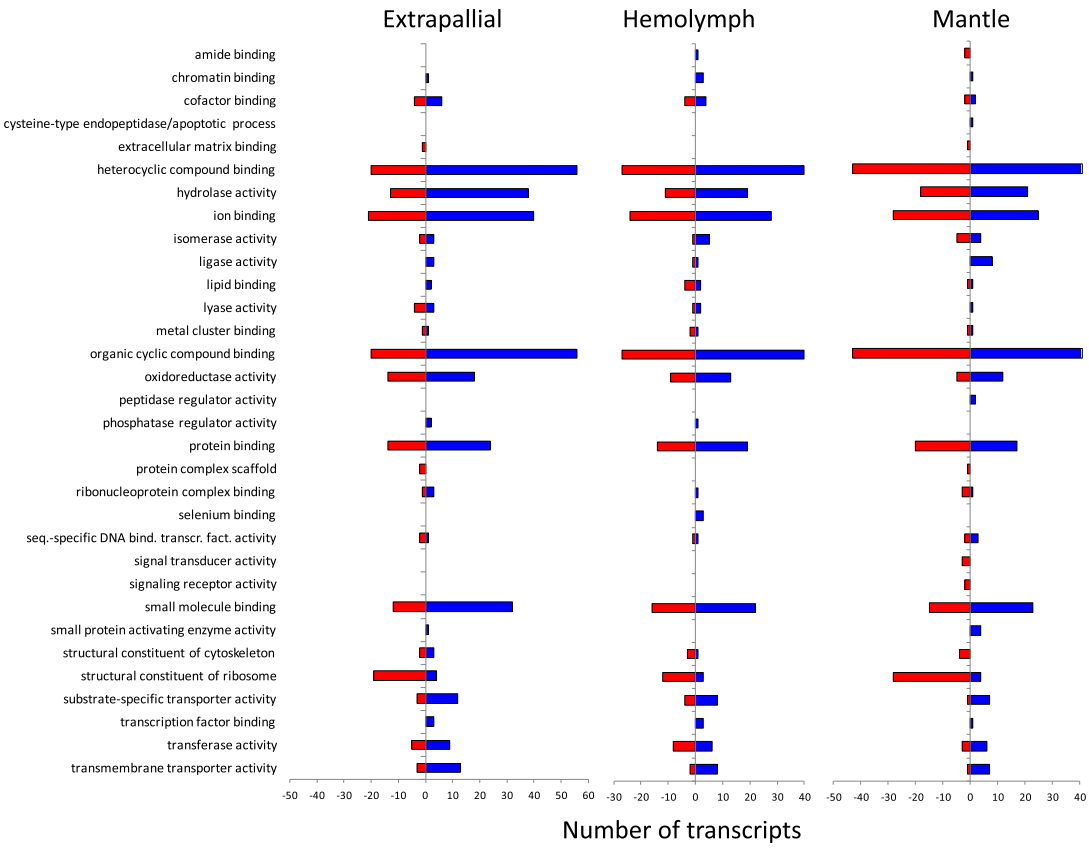

Fig. 2. Molecular functions (GO terms, level 3) regulated in different tissues from infected clams as compared to healthy individuals. The number of down-regulated (negative) and up-regulated (positive) transcripts is given.

The distribution of GO terms in the mantle was more even across up- and down-regulated transcripts.

Molecular function GO terms also showed a more pronounced differential display among transcripts regulated in the extrapallial fluid (Fig. 2), with a generally higher representation among up-regulated transcripts for GO terms as diverse as "hydrolase activity" (GO:0016787, 13 down- and 38 up-regulated transcripts), "ion binding" (GO:0043167, 21 down- and 40 up-regulated transcripts), "organic cyclic compound binding" (GO:0097159, 20 down- and 56 up-regulated transcripts), "substrate-specific transporter activity" (GO:0022892, 3 down- and 12 up-regulated transcripts) and "transmembrane transporter activity" (GO:0022857, 3 down- and 13 up-regulated transcripts). The later 2 terms were also over-represented among up-regulated transcripts in the hemolymph and mantle. The only molecular function GO term that was over-represented among down-regulated transcripts was "structural constituent of ribosome" (GO:0003735).

Enrichment analysis showed several GO terms to be significantly over-represented among transcripts down-regulated in the extrapallial fluid of diseased clams including "retinol metabolic process" (GO:0042572; 58 fold enrichment), "methionine synthetic process" (GO:0009086, 39 fold enrichment) and "one-carbon metabolic process" (GO:0006730, 15 fold enrichment) (Fig. 3). The terms "proton-transporting ATP synthase complex coupling factor" (GO:0045263) and "nucleus" (GO:0005634) were over-represented (3 and 18 fold enrichment, respectively) among transcripts up-regulated in the extrapallial fluid (data not shown). In the hemolymph, only the term "arginine kinase activity" (GO:0004054) was over-represented (158 enrichment fold) among down-regulated transcripts and there were no statistically-significant enriched GO terms among up-regulated transcripts (data not shown). Finally, only the term "lysozyme activity" (GO:0003796) was enriched (36 fold) among down-regulated transcripts in the mantle while "small protein activating enzyme activity" (GO:0008641) was the only term enriched (179 fold) among up-regulated transcripts in the mantle (data not shown).

3.2. Immune genes differentially regulated in diseased clams

Several immune-related genes were regulated in BRD-infected clams (Tables 1–3; Supplementary Tables S1 to S6). Up-regulated genes in the extrapallial fluid included several pathogen recognition receptors (PRR) such as lectins (tandem repeat galectin, sialic-acid binding lectin, c-type lectin, and mannose-binding lectin), complement factor-related proteins and hemagglutinins (Table 1, Supplementary Table S1). Up-regulated transcripts also included proteases (serine and cysteine proteases), a protease inhibitor and several apoptosis-related transcripts (death-associated protein and the anti-apoptotic protein bcl-2). Down-regulated transcripts in the extrapallial fluid also included different variants of PRR proteins (complement factor-related proteins, lectins, thioester-containing proteins). Notably, several antimicrobial factors (defensins, lysozyme) were down-regulated in the extrapallial fluid of infected clams (Table 1 and Supplementary Table S2). Down-regulated transcripts also included 2 homologs of tumor-necrosis factor (TNF) and an inhibitor of apoptosis protein.

As for the extrapallial fluid, regulated transcripts in the hemolymph included several antimicrobial proteins, PRR, and apoptosis regulation proteins (Table 2, Supplementary Tables S3 and S4). Cysteine proteases were highly represented among up-regulated transcripts with a cathepsin displaying a 13.6 fold up-regulation in diseased clams as compared to healthy individuals. Transcripts

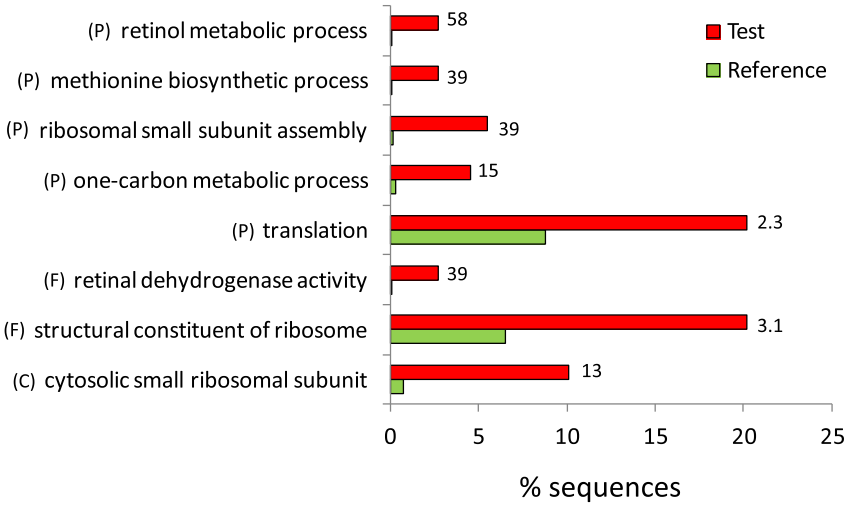

Fig. 3. GO terms (reduced to the most specific terms) significantly enriched among down-regulated transcripts in the extrapallial fluid of diseased clams. Values shown next to the bars represent the ratio test/reference (% sequences among down-regulated transcripts versus % sequences on the entire oligoarray for a given GO term). (P): Biological process, (F): molecular function, (C): cellular component.

up-regulated in hemolymph also included a big defensin, lysozyme and apoptosis-related proteins. Down-regulated transcripts also included several PRR (complement-related factor, hemagglutinin, thioester-containing proteins) and a thaumatin-like protein (4 fold down-regulation in diseased clams).

The regulation of immune-related transcripts was less pronounced in the mantle as compared to hemocytes from the extrapallial fluid or hemolymph (Table 3, Supplementary Tables S5 and S6). Up-regulated transcripts in diseased clams included a sialic-binding lectin (2.5 fold), a cysteine protease (cathepsin), a serine protease inhibitor and a big defensin. Down-regulated transcripts in the mantle of infected clams included a thaumatin-like protein (2.8 fold down-regulation), complement factor-like proteins, a TNF-like protein and lysozyme.

3.3. Genes related to biomineralization

Most strong regulation of transcripts associated with calcium transport and biomineralization was noticed in the extrapallial fluid of infected clams (Table 1) as compared to the hemolymph (Table 2) and mantle (Table 3). Up-regulated transcripts in the extrapallial fluid of diseased individuals included the extracellular

Table 1
Representative transcripts significantly regulated in the extrapallial fluid of diseased clams.

Upregulated in diseased clams				Downregulated in diseased clams			
Transcript	Description	GeneBank	Fold	Transcript	Description	GeneBank	Fold
Immune system				**Immune system**			
r31997	Tandem repeat galectin	ACA09732	10.6	r35701	c1Q domain containing protein 1q77	CBX41726	−5.1
r33988	c1Q-domain-containing protein	CBX41719	3.8	r15412	Complement factor b-like	XP_003218047	−3.8
r32328	Kazal-type serine proteinase inhibitor	ACU83227	3.8	r30937	Cathepsin l	BAK02675	−3.1
r36251	Complement factor b-like protein	ACQ91095	3.5	i279	Big defensin 1	AEE92785	−2.9
r25219	Cathepsin l preproprotein	XP_003148162	3.0	r24480	Big defensin 2	AEE92786	−2.9
r15086	Cysteine proteinase	AAB17051	2.3	n2902	Hemagglutinin amebocyte aggregation factor	Q01528	−2.7
r35492	Sialic acid-binding lectin	ACU83226	2.3	r15744	Sialic acid-binding lectin	ACU83226	−2.7
n5225	Serine protease	ADA61200	2.0	r25646	c-type lectin	ACU83213	−2.5
r31290	c-type lectin domain family member a-like	XP_003123415	2.0	r31805	Achain crystal structure of tapes japonica lysozyme	BAC15553	−2.2
r25647	Death-associated protein	XP_002732013	1.9	r25824	c-type lectin	XP_001630495	−2.1
r37090	Hemagglutinin amebocyte aggregation factor-like	CAC38786	1.9	r12009	Big defensin	ADM25826	−2.1
r31843	bcl-2 protein	XP_002740789	1.8	r23729	Thioester-containing protein	BAE44110	−1.9
r36816	Mannose receptor c type 1-like	XP_001630513	1.7	r24654	Lipopolysaccharide-induced tnf-alpha factor	ADX31291	−1.8
r36875	Astacin-like squid metalloprotease type iii	BAC16239	1.7	r35688	Tumor necrosis factor-like protein	ABZ89640	−1.8
r19343	Defensin precursor	XP_002918413	1.6	r33768	Inhibitor of apoptosis protein 1	XP_308529	−1.7
Calcium binding and biomineralization				**Calcium binding and biomineralization**			
r37442	Dermatopontin 3	EGD73545	6.3	r34457	Extracellular matrix protein ecm 18	XP_001654057	−4.3
r10111	Shell matrix protein	P86860	2.8	r36339	Sarcoplasmic calcium-binding protein	BAA25310	−2.2
r24901	Von willebrand factor c and egf domains	XP_002599939	2.7	r36064	b-cell translocation gene 1	ACH92125	−2.0
r26896	Sparc protein	CAJ38815	2.5	n3570	Calcium homeostasis endoplasmic reticulum protein	XP_002260516	−2.0
r24422	Perlucin-like protein	EFN68216	2.3	r21847	Conserved hypothetical protein: chitin binding	XP_002425293	−1.9
r30594	Chitin binding peritrophin-a domain protein	EFV57854	2.1	i460	Collagen alpha-1 chain-like	XP_002936246	−1.9
r25766	Stanniocalcin-like protein	ABW90687	2.1	r10552	Sarcoplasmic calcium-binding protein	BAA75223	−1.8
r30808	Ependymin-like protein	XP_002596851	1.8	r10624	Carbonic anhydrase	NP_001016431	−1.8
r32630	Calcium-binding ef-hand-containing protein	BAD16599	1.8	r12083	Troponin skeletal muscle	XP_001339131	−1.5
r38209	Ca2+ sensor	YP_002006966	1.5				

Table 2
Representative transcripts significantly regulated in the hemolymph of diseased clams.

Upregulated in diseased clams				Downregulated in diseased clams			
Transcript	Description	GeneBank	Fold	Transcript	Description	GeneBank	Fold
Immune system				**Immune system**			
r25198	Cathepsin l1	XP_002935514	13.6	r35701	c1q domain containing protein 1q77	CBX41726	−7.3
r12369	Big defensin	ADM25826	8.9	n1091	Kazal-type serine proteinase inhibitor	ACU83227	−5.8
n1222	c1q-domain-containing protein	CBX41719	3.7	r15412	Complement factor b-like	XP_003218047	−4.6
r36251	Complement factor b-like protein	ACQ91095	3.5	r31874	Thaumatin-like protein	XP_002887571	−4.0
r25219	Cathepsin l preproprotein	XP_003148162	3.3	i279	Big defensin 1	AEE92785	−3.2
r15086	Cysteine proteinase	AAB17051	2.8	r24480	Big defensin 2	AEE92786	−2.7
r37090	Hemagglutinin amebocyte aggregation factor-like	CAC38786	2.3	r27163	Hemagglutinin amebocyte aggregation factor	Q01528	−2.4
n5818	Complement component q subcomponent-like 2	EDL39796	2.2	r31805	Tapes japonica lysozyme	BAC15553	−2.2
n5121	Digestive cysteine protease intestain	ACP18843	1.9	r23729	Thioester-containing protein	BAE44110	−1.7
r24571	Cathepsin l2	XP_002130162	1.9	r37213	Inhibitor of apoptosis protein 1	AAL46972	−1.7
r31843	bcl-2 protein	XP_002740789	1.8	r36102	Inhibitor of apoptosis protein (agap007291-pa)	XP_308529	−1.6
r30840	Achain crystal structure of tapes japonica lysozyme	ACU83237	1.8	r35688	Tumor necrosis factor-like protein	ABZ89640	−1.6
r37696	Cysteine protease inhibitor	XP_002322604	1.7	n1219	Peptidoglycan binding domain-containing protein	XP_002733096	−1.5
r19102	Tandem repeat galectin	ACA09732	1.5	n1842	Cell adhesion protein	XP_002607038	−1.5
r6350	Programmed cell death 7-like	XP_001509679	1.5	n2913	Allograft inflammatory factor	ACU83234	−1.5
Calcium binding and biomineralization				**Calcium binding and biomineralization**			
r37442	Dermatopontin 3	EGD73545	2.1	r38777	DEC-3 [Lymnaea stagnalis]	BAD16599	−2.3
i309	Collagen alpha-1 chain-like	XP_002936246	2	r28553	Sarcoplasmic calcium-binding protein	BAA75223	−2.2
i374	Calmodulin	ACT88125	1.9	n1235	Alpha 2 type i collagen	XP_002190214	−2.1
				n3827	Centrin 3-like protein	NP_001161514	−1.9
				r35095	Kielin chordin-like	XP_002752096	−1.8
				r24422	Perlucin-like protein	EFN68216	−1.5

matrix protein dermatopontin (6.3 fold), a shell matrix protein, a perlucin, a chitin-binding and other proteins involved in biomineralization. On the other hand, down-regulated transcripts in the extrapallial fluid included an extracellular matrix protein (ecm 18, 4.3 fold down-regulation), a chitin-binding protein, a carbonic anhydrase and several proteins involved in calcium fixation. Up-regulated transcripts in the hemolymph of infected clams also included dermatopontin (2.1 fold) and the extracellular matrix protein collagen, while down-regulated transcripts included perlucin and several calcium-binding proteins. In mantle, up-regulated transcripts included the phenoloxidase laccase (2.9 fold), perlucin and a calcium-binding protein, while down-regulated transcripts included the shell matrix protein galaxin and a chitin-binding protein.

3.4. Differential expression across different tissues

Most regulated transcripts were tissue-specific (Fig. 4, Supplementary Figures S2 and S3). For instance, only 176 (26%) of the 683 transcripts up-regulated in the extrapallial fluid of infected clams were shared with the hemolymph, and merely 6% (40 transcripts) with those up-regulated in the mantle. Only 3% of the transcripts were shared among all 3 tissues. Similarly, 20% of transcripts down-regulated in the extrapallial fluid were also down-regulated in the hemolymph of infected clams, and only 3% were shared between the extrapallial fluid and mantle while 6% were shared between all tissues. Interestingly, some transcripts displayed contrasting trends between different tissues (Fig. 4, Supplementary Figures S4 to S7). For instance, 14 transcripts were up-regulated the extrapallial fluid while they were down-regulated in the hemolymph (9 transcripts including a serine protease inhibitor and a perlucin-like protein) or the mantle (5 transcripts including a chitin binding protein and several calcium-binding proteins) (Supplementary Figure S4). Similarly, 7 transcripts were down-regulated in hemolymph and up-regulated in the mantle of infected clams including a serine protease inhibitor and 2 calcium-binding proteins (Supplementary Figure S5). A total of 7 transcripts were up-regulated in hemolymph and down-regulated in mantle (4 transcripts including a cathepsin and a vitellogenin receptor) or in the extrapallial fluid (3 transcripts with no blast hit) (Supplementary Figure S7).

Table 3
Representative transcripts significantly regulated in the mantle of diseased clams.

Upregulated in diseased clams				Downregulated in diseased clams			
Transcript	Description	GeneBank	Fold	Transcript	Description	GeneBank	Fold
Immune system				**Immune system**			
r35492	Sialic acid-binding lectin	ACU83226	2.5	r31874	Thaumatin-like protein	XP_002887571	−2.8
r25198	Cathepsin l1	XP_002935514	2.5	r15412	Complement factor b-like	XP_003218047	−2.6
n1091	Kazal-type serine proteinase inhibitor	ACU83227	2.2	r31805	Achain crystal structure of tapes japonica lysozyme	BAC15553	−1.8
n3078	Pathogenesis-related protein 5 precursor	XP_002890328	1.6	r28380	c-type mannose receptor 2-like	XP_002593722	−1.8
r30394	Big defensin	ADM25826	1.6	n1800	Cathepsin l1 cysteine protease	ADC52430	−1.7
				r36995	Tandem repeat galectin	ACA09732	−1.7
				r35688	Tumor necrosis factor-like protein	ABZ89640	−1.6
				i6	c1Q domain containing protein 1q67	CBX41716	−1.6
Calcium binding and biomineralization				**Calcium binding and biomineralization**			
r11956	Laccase 1	EFX81873	2.9	r36544	Galaxin [Montipora capitata]	ABV24967	−1.9
r28553	Sarcoplasmic calcium-binding protein	BAA75223	2.0	r30594	Chitin binding peritrophin-a domain protein	EFV57854	−1.7
r35668	Perlucin-like protein isoform b	ACU83225	1.6	r38209	Ca2+ sensor	YP_002006966	−1.6

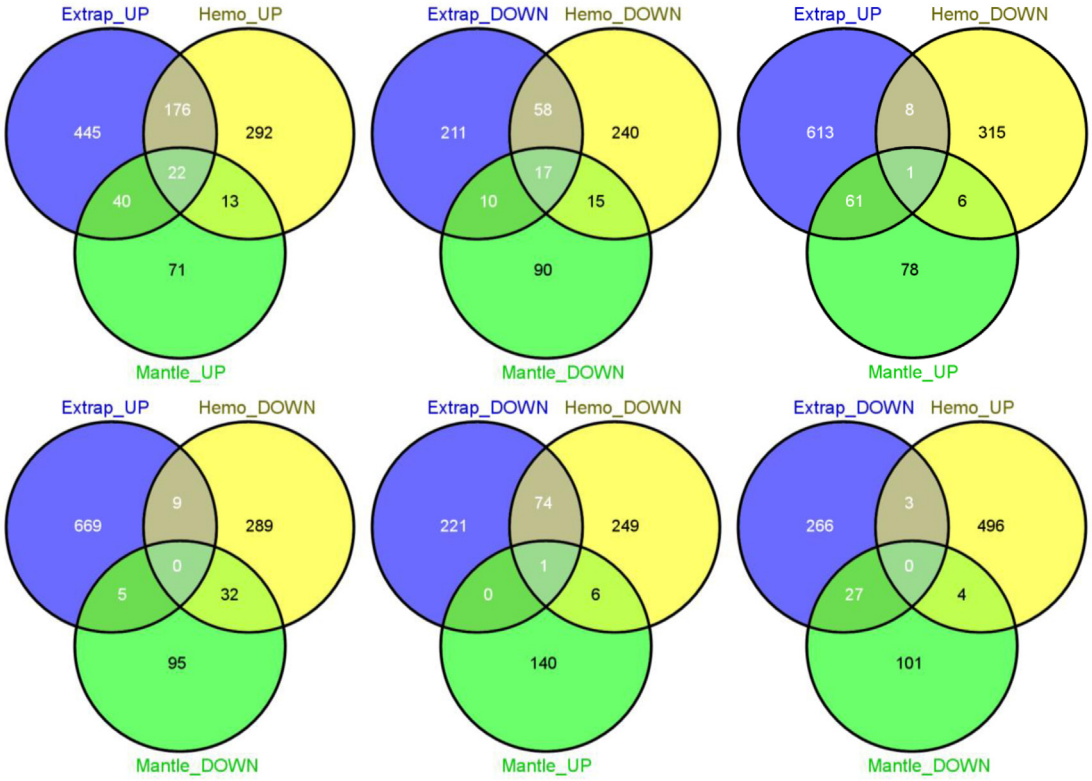

Fig. 4. Distribution of regulated transcripts across the various tissues (extrapallial fluid, hemolymph and mantle) investigated. Most regulated transcripts were tissue-specific. See Supplementary Figures S2 to S7 for details.

4. Discussion

The main objective of this study was to identify molecular changes associated with BRD in Manila clam. Results depicted some integrative aspects of clam responses to the disease and showed significant alterations in the transcription levels of immune-related genes and in genes involved in the biomineralization process. Since BRD is a shell disease, we focused our study on the comparison of transcriptomic changes in mantle (shell-producing organ), extrapallial fluid (site of biomineralization and focal point of the infection) and hemolymph ("classical" immune fluid). Interestingly, most changes were noticed in the extrapallial fluid, an often overlooked peripheral compartment that contains potent defense factors. These results confirm the importance of the extrapallial fluid as a first line of defense against *V. tapetis*, in agreement with previous studies targeting cellular and humoral immune factors associated with BRD [21–23].

The contrasting findings between hemolymph and the extrapallial fluid (Fig. 4) are very intriguing given the fact that both body fluids are made up of hemocytes that share common morphological and functional characteristics [25]. It is therefore plausible that transcriptomic changes measured in the current study represent signatures of focal (extrapallial fluid) and systemic (hemolymph) responses to the infection. Some transcripts (perlucin, serine protease inhibitor) displayed opposite trends between both fluids highlighting a spatially-targeted response to the infection. Overall, results showed that BRD is associated with significant changes in the transcription levels of molecules involved in pathogen recognition (e.g. lectins, c1q-like proteins) and killing (defensin, lysozyme), apoptosis regulation (death-associated protein, bcl-2, TNF-like protein) and in biomineralization (shell matrix protein, perlucin, galaxin, chitin- and calcium-binding proteins).

Results also showed that different transcripts belonging to the same functional group sometimes displayed opposing trends within the same tissue. This is particularly the case for members of the C1q domain-containing proteins (C1QDC and their sialic-acid binding lectin relatives) which were present among transcripts up-regulated in the extrapallial and hemolymph of infected clams while other members of the same group were down-regulated (Tables 1 and 2, Supplementary Tables S1–S4). C1qDCproteins have been previously shown to represent a highly diverse group of molecules playing an important role as pathogen recognition receptors [41–44]. For instance, previous studies reported the presence in the mussel *Mytilus galloprovincialis* of 168 different transcripts of C1qDC, many of which showing differential expression following challenge with Gram-positive or -negative bacteria [37,41,42,45]. Similar findings were also reported in the European clam *Ruditapes decussatus* by Leite et al. [46] who described opposing trends of various C1qDC transcripts in clams infected with *Perkinsus olseni*, while a recent study in *R. philippinarum* showed a down-regulation of 3 members of the C1q family in hemocytes following in vitro exposure to *Vibrio alginolyticus* [37]. It is therefore plausible that some transcripts are up-regulated while others are switched down providing a tailored response to BRD in Manila clam. Overall, these findings highlight the need for further investigations to generate a better understanding of the specific role of different C1qDC transcripts during BRD development, and particularly their possible interactions with the pathogen *V. tapetis*.

While several strongly regulated transcripts were not annotated, the most up-regulated transcript in the extrapallial fluid of diseased clams was a tandem repeat galectin (10.6 fold, Table 1). This transcript is weakly related to the tandem-repeat galectin MCGal (matching NCBI ACA09732 with an e-value = 1.6E-5) previously described in *R. philippinarum* [47]. This transcript was not

regulated in hemolymph or mantle suggesting that this it may be produced by specific hemocytes present in the extrapallial fluid or is part of the focal response to the infection. Results also showed the regulation of other transcripts strongly matching MCGal such as transcript r37255 (e-value = 6.7E-161) and r19102 (e-value = 4.0E-20) which were up-regulated in the extrapallial fluid (1.9 fold) and the hemolymph (1.5 fold), respectively, but not in the mantle. These galectins may contribute to the immune response of *R. philippinarum* to the infection as proposed for MCGal which was found to be up-regulated after clam challenge with pathogens including *V. tapetis* and *Perkinsus* spp [30,47]. In fact, galectins are well known to contribute to immunity by binding to and opsonising microbial pathogens [14,48] including *V. tapetis* in the case of MCGal [47].

Our results identified several defensins that showed different expression patterns in various tissues. Defensins are a large group of small antimicrobial peptides and represent major actors in innate immunity [49]. Their involvement in clam response to BRD was previously suggested by Adhya et al. [50] and Jeffroy et al. [30] who reported up-regulation in defensin transcription in Manila clams naturally-infected or experimentally-injected with *V. tapetis*. Various defensins have been characterized in different bivalve species [15,50,51] where they display a vast diversity in their structure, biological properties and functions, and in their tissue distribution and expression. In the oyster *Crassostrea gigas*, at least 6 defensins have been identified so far, mainly expressed in hemocytes [15,52,53]. Defensins can be constitutively expressed [52], or induced in response to infection, as in scallop [54] and clams [30,50,55]. Defensins from *C. gigas* exhibit high activities against Gram-positive bacteria but low activity against fungi [15], whereas big defensins from the scallop *Argopecten irradians* and the horseshoe crab *Tachypleus tridentatus* exhibit strong fungicidal activities [54,56]. In our study, an alignment allow the identification of 7 unique defensin sequences (data not shown). Two of these (represented by the probes i279 and n5549) were down-regulated in both hemolymph and extrapallial fluid (fold change varies from −1.5 to −3.17). Another defensin (represented by the probe n1572) was up-regulated in the 3 tested tissues (fold varies from 1.5 to 2.05). An additional defensin was down-regulated in hemolymph while three others were up-regulated in both hemolymph and extrapallial fluid. Among these, transcript r12369 showed an 8.9 fold over-expression in the hemolymph of diseased clams (Table 2) suggesting its involvement in the systemic response to BRD. These results are not surprising since our previous studies in the hard clam *Mercenaria mercenaria* also showed contrasting regulation of 2 defensins (a big and a hemocyte defensin) in different tissues following exposure to the labyrithulomycete pathogen QPX [55].

Another group of transcripts highly regulated in BRD-infected clams was cathepsins. The sequence r25198 (cathepsin L1, XP_002935514, e-value 9.7E-26) was the most highly up-regulated transcript in the hemolymph (13.6 fold, Table 2) and was also up-regulated in the mantle (2.5 fold, Table S5) but not in the extrapallial fluid. Other cathepsins (e.g. XP_002708337, e-value 2.6E-37) were either up- or down-regulated across different tissues suggesting significant changes in protein metabolism in response to the infection. Cathepsins are a large groups of proteases that differ by their structure, catalytic mechanism, and the type of proteins they cleave. For example, members of the cathepsin L group are cysteine proteases that were suggested to play an important role in immunity after they have been shown to be up-regulated following bacterial exposure in different bivalve species including the pearl oyster [57] and the razor clam [58]. In Manila clam, Menike et al. [59] described significant up-regulation in the transcription level of a cathepsin D in hemocytes following intramuscular injection of *V. tapetis*. Cysteine proteases, including members of the cathepsin L family, are also strongly implicated in the regulation of apoptosis [60,61] and changes in their expression was associated with the regulation of several apoptosis-related proteins particularly in the extrapallial fluid (bcl-2, inhibitor of apoptosis protein, TNF-like protein) and hemolymph (bcl-2, programmed cell death 7-like, inhibitor of apoptosis protein) suggesting an orchestrated regulation of pro- and anti-apoptotic processes during BRD development. A similar regulation of transcripts related to apoptosis was recently described in *R. philippinarum* hemocytes exposed to *V. alginolyticus* in vitro [37].

The regulation of proteases was also associated with significant changes in the transcription levels of protease inhibitors. This was particularly the case for several transcripts matching serine protease inhibitors that were up-regulated in the extrapallial fluid (up to 3.8 fold increase, none was down-regulated) and the mantle (2.2 fold) of infected clams. In contrast, serine protease inhibitors were strongly down-regulated (−5.8 fold) in the hemolymph of infected clams in agreement with the results of Jeffroy et al. [30], while cysteine protease inhibitors were up-regulated (1.7 fold). These findings provide further evidence of orchestrated response to the infection across different tissues. Protease inhibitors may play a dual role by protecting the host from the deleterious effect of over-expressed protease activities [62,63], and by providing protection against proteases produced by the invading microorganisms. With that regard, there is strong evidence for the involvement of host protease inhibitors as determinant factors for resistance to infectious diseases in several animal taxa [64,65] including bivalves [66,67].

Serine proteases and their inhibitors are also strongly involved in the phenoloxidase cascade and the melanization process (see reviews by [68,69]). Melanization response is a major trait of BRD in Manila clams and is usually associated with major rearrangements of shell matrix deposition [27]. *V. tapetis* alters the shell biomineralization process by colonizing and disrupting the organic matrix that supports shell growth [4,27,70]. Clam responds to the infection in two steps, first by embedding *V. tapetis* in melanized shell matrices, and later by covering the abnormal organic deposit with new calcified shell layers in a mechanism similar to the nacrezation process during pearl formation [27]. A recent small scale transcriptomic study has pointed out potential implication of genes involved in shell repair process during BRD, such as carbonic anhydrase [30]. In the present study, several genes involved in calcium binding and biomineralisation (Tables 1–3) showed major regulation either in hemocytes or in the mantle of diseased clams. Most of these changes were noted in hemocytes for the extrapallial fluid. For example, perlucin-like transcripts (which contain C-type lectin-like domains) were up-regulated in the extrapallial fluid and the mantle of diseased clams (down-regulated in hemolymph). Perlucin nucleates the growth of calcium carbonate crystals in mollusks [71,72] and was suggested to play a dual role as an organic support for biomineralization and as a potential defense molecule against pathogenic microorganisms [73]. Our results also showed significant up-regulation in hemocytes of genes involved in shell matrix synthesis in mollusks [74], such as dermatopontin (hemolymph and extrapallial fluid), chitin binding peritrophin-a domain protein, sparc, ependymin-related protein and shell matrix proteins (extrapallial fluid), and calmodulin (hemolymph). In pearl oysters and blue mussels, key shell proteins of the nacreous matrix and periostracum interact strongly with chitin and some of these (Pif, BMSP) present a chitin binding peritrophin-a domain that is thought to play a role in structuring the matrix [75,76]. As mentioned above, *V. tapetis* disrupts the periostracum leading to the development of a melanized deposit [4,27,70]. Melanin biosynthesis is mediated by a diverse group of enzymes designated

under the generic name of phenoloxidases that encompass laccases. In the present study, a laccase transcript was significantly up-regulated in the mantle of diseased clams suggesting its role in the melanization process. This was accompanied by a down-regulation of galaxin, a major component of the organic matrix of the exoskeleton in corals [77].

Interestingly, several transcripts with the GO terms "lipid metabolic process" or "lipid transport" were up-regulated (none down-regulated) in hemocytes from the extrapallial fluid (Supplementary Table S1) and these may be part of the shell repair process induced by BRD. For instance, previous studies demonstrated the role of phospholipids in hemocytes of the snail *Helix pomatia* in the calcification process during shell repair [78,79]. In this context, the amorphous calcium carbonate (ACC) is packed in lipid vesicles that stabilize ACC by isolating it from the aqueous environment and allowing its delivery to the site of mineralization [80]. A similar mechanism was also suggested for the building of coral exoskeletons [81]. In oysters, Mount and collaborators [82] showed that a specialized class of granulocytes migrate through the mantle epithelium to deposit calcium carbonate crystals at the mineralization front in the extrapallial space. These crystals are released as lipid-bound exosomes at the site of shell formation [83]. Therefore, change in lipid metabolism and transport reported here in hemocytes may in fact reflect changes in shell deposition rate or structure. Overall, the fact that the regulation in diseased clams of genes potentially implicated in biomineralization was noted not only in mantle but also in hemocytes from hemolymph and particularly from the extrapallial fluid suggests a complex modification of the secretion of shell matrices during the development of BRD. The findings are in line with microscopic observations and biochemical analyses showing major shifts in the structure and composition of clam shell material during BRD [17,84].

In conclusion, this study represents a first attempt to explore transcriptomic changes in Manila clam in response to BRD. A large number of candidate genes was identified including several genes involved in defense response and biomineralization. The results highlight the role of the extrapallial fluid in clam response toward BRD. Additional experiments are needed to further characterize molecular components involved in *R. philippinarum* interactions with *V. tapetis*. Specifically, further experiments should compare gene expression profiles in susceptible and resistant clam broodstocks. Specific experiments should also be designed to probe the exact molecular function of most promising transcripts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2014.05.022.

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