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# Journal Pre-proof

A proteomic study of resistance to Brown Ring disease in the Manila clam, *Ruditapes philippinarum*

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**Credit Author Statement**

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Journal Pre-proof

1 A PROTEOMIC STUDY OF RESISTANCE TO BROWN RING DISEASE IN THE MANILA  
2 CLAM, *Ruditapes philippinarum*.

3  
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10  
11 ABSTRACT

12 Marine mollusk aquaculture has more than doubled over the past twenty years, accounting for over  
13 15% of total aquaculture production in 2016. Infectious disease is one of the main limiting factors to  
14 the development of mollusk aquaculture, and the difficulties inherent to combating pathogens through  
15 antibiotic therapies or disinfection have led to extensive research on host defense mechanisms and host-  
16 pathogen relationships. It has become increasingly clear that characterizing the functional profiles of  
17 response to a disease is an essential step in understanding resistance mechanisms and moving towards  
18 more effective disease control. The Manila clam, *Ruditapes philippinarum*, is a main cultured bivalve  
19 species of economic subject to Brown Ring Disease (BRD), an infection induced by the bacterium  
20 *Vibrio tapetis*.

21 In this study, juvenile Manila clams were subjected to a 28-day controlled challenge with *Vibrio*  
22 *tapetis*, and visual and molecular diagnoses were carried out to distinguish two extreme phenotypes  
23 within the experimental clams: uninfected ("RES", resistant) and infected ("DIS", diseased) post-  
24 challenge. Total protein extractions were carried out for resistant and diseased clams, and proteins were  
25 identified using LC-MS/MS. Protein sequences were matched against a reference transcriptome of the  
26 Manila clam, and protein intensities based on label-free quantification were compared to reveal 49  
27 significantly accumulated proteins in resistant and diseased clams. Proteins with known roles in  
28 pathogen recognition, lysosome trafficking, and various aspects of the energy metabolism were more  
29 abundant in diseased clams, whereas those with roles in redox homeostasis and protein recycling were  
30 more abundant in resistant clams.

31 Overall, the comparison of the proteomic profiles of resistant and diseased clams after a month-long  
32 controlled challenge to induce the onset of Brown Ring disease suggests that redox homeostasis and  
33 maintenance of protein structure by chaperone proteins may play important and interrelated roles in  
34 resistance to infection by *Vibrio tapetis* in the Manila clam.

## 35 1. INTRODUCTION

36 Mollusks represent over a fifth of the global aquaculture market, accounting for USD 29.2 billion  
37 in 2016 of which the most heavily traded species are oysters, clams, scallops, and mussels.  
38 Originating from the Asian Pacific coast, the Manila clam, *Ruditapes philippinarum* has become  
39 the second major cultured bivalve in the world, with over 4.4 million tons per year produced  
40 worldwide [1]. This species was introduced to the French Atlantic coast for aquaculture  
41 diversification in the 1970s [2], and following a rapid increase in clam production, mortality  
42 events became increasingly frequent and severe, eventually leading to the closure of many clam  
43 production parks [3]. The mass mortality episodes were subsequently associated to Brown Ring  
44 disease (BRD) [4,5], a chronic extra-pallial infection caused by *Vibrio tapetis*. After initial  
45 proliferation of the bacteria in the extra-pallial compartment, diseased clams manifest abnormal  
46 conchiolin deposits along the inner surface of the shell. In severe infections, the pathogen may  
47 cause lesions in the mantle and penetrate the hemolymph, in which case septicemia and death  
48 occur within 4-5 days [6,7].

49 In France, BRD continues to negatively impact production and prevalence can reach 80 - 100 %  
50 [8] along the Northern Atlantic coast. While the severity of the disease and the virulence of its  
51 etiological agent are known to be largely dependent on a number of environmental factors,  
52 namely temperature and salinity, bivalves have a number of sophisticated stress and immune  
53 response mechanisms, as well as a highly specific innate immune system on which they rely to  
54 directly combat infection [9,10]. As marine bivalves lack an adaptive immune system, the innate  
55 genomic component of their immune system plays an essential role in mitigating the host  
56 response to pathogens.

57  
58 Invertebrate innate immunity relies on a number of pathogen recognition factors that trigger  
59 signaling pathways involved in hemocyte recruitment, phagocytosis, and the production of a wide  
60 range of antimicrobial compounds for host defense. While resistance to infection in bivalves  
61 initially depends on the ability of mucosal interfaces to impede pathogen entrance into the host,  
62 circulating hemocytes from fluids (such as hemolymph and extra-pallial fluids) and bioactive  
63 molecules in the plasma are responsible for mediating the secondary host response through  
64 phagocytosis and direct bacterial neutralization by antimicrobial effectors [11,12]. Interestingly,  
65 clams have been shown to recover from BRD through shell repair processes, leading to the

66 investigation of this resistant phenotype in several populations [13]. Previous gene expression  
67 and transcriptomic studies on the Manila clam have led to the assembly of a transcriptome and  
68 have shed light on several factors such as pathogen recognition and killing, modulation of  
69 hemocyte cytoskeleton, regulation of apoptosis, and bio-mineralization that are likely to play key  
70 roles in the innate immune response against bacterial infections [4–8,14–19]. The factors  
71 influencing virulence and highlighting the dynamics of the infection process leading to Brown  
72 Ring disease in the Manila clam are increasingly well described [8,20–23], and it is has become  
73 clear that the interactions between host and pathogen during infection leads to a complex  
74 remodeling of the molecular framework of both organisms, highlighting the importance of  
75 understanding the changes in gene expression as well as those occurring on the proteomic level.  
76 In addition, several studies focusing on *Vibrio*-induced expression of immune-related genes in *R.*  
77 *philippinarum* suggest a tendency towards a downregulation of the inflammatory response and an  
78 upregulation of genes related to homeostasis in this species, insisting on the importance of  
79 investigating the molecular mechanisms at play to identify markers of resistance [24,25]. The  
80 growing number of genomic and transcriptomic resources available for this host species have  
81 unveiled particularly high levels of polymorphism, a factor that may mitigate the observed  
82 functional variability in the immune response to pathogens [26,27], though to date there remains  
83 a significant knowledge gap surrounding the functional response of the Manila clam to infection  
84 with *Vibrio tapetis*, particularly in disease-resistant clams.

85 The present study aims to characterize the proteomic profiles of resistant and susceptible Manila  
86 clams following infection with *Vibrio tapetis*. By comparing the functional response in these two  
87 extreme phenotypes, we seek to shed light on the factors responsible for resistance to pathogens  
88 in this invertebrate species.

89

## 90 2. MATERIALS AND METHODS

### 91 2.1. *Experimental design and sampling*

92 Juvenile *R. philippinarum* (average shell length 12.37 mm) from a cohort of mixed families  
93 produced at the SATMAR hatchery (Marennes, France) were acclimated for 12 days in aerated  
94 seawater tanks at 14°C. An experimental group (n=1200) was exposed to air for 8 hours at 14°C  
95 to facilitate valve opening, then placed in a shallow tank and injected into the pallial cavity  
96 (without damaging the mantle epithelium) with 50 µL of *V. tapetis* suspension (strain  
97 CECT4600<sup>T</sup>) prepared in filter-sterilized sea water (FSSW) containing  $8.2 \times 10^6$  bacteria.mL<sup>-1</sup>  
98 ( $4.1 \times 10^5$  bacteria injected per clam). A first control group (C1; n=300) was injected with 50 µL  
99 of FSSW, and a second control group (C2; n=300) received no treatment. All clams (injected as  
100 well as not injected) were then kept in dry conditions for at least 6 hours to ensure that the  
101 injected clams retained the fluid, then returned to separate tanks in a thermoregulated room at  
102 14°C with no food and constant aeration for four weeks, according to a standardized protocol  
103 established by Paillard & Maes [28]. As BRD is a chronic infection localized in the extra-pallial  
104 compartment, injection into the pallial cavity mimics the natural infection process, whereas  
105 injecting tissues results in rapid septicemia for *V. tapetis* injections, or tissue disruption in the  
106 case of sterile filtered sea water injection into the tissues. Dead clams, when they occurred, were  
107 recorded daily and removed from the tanks. The seawater remained unchanged throughout the  
108 duration of the experiment. After the four-week incubation period, all clams were sampled for  
109 whole weight, shell weight, shell length, and each clam was individually opened with a cleaned  
110 scalpel over a tube, allowing whole tissues and fluids to be collected together, after which the  
111 sample was flash frozen in liquid nitrogen and stored at -80°C.

112

### 113 2.2. *Diagnostic methods*

114 For visual diagnosis, shells were observed under a binocular magnifier to identify and quantify  
115 the extent index of Brown Ring disease according to the classification system described by  
116 Paillard & Maes (1994). For molecular diagnosis, DNA from whole-body tissue and fluid  
117 samples was analyzed. Briefly, the samples were homogenized in a volume of phosphate buffer  
118 saline (PBS; pH = 7) based on tissue weight, for a final concentration of 0.25 mg/µL tissue in the  
119 buffer. Ceramic beads were added to each sample and mechanical tissue disruption was done  
120 using 2 cycles of 20 seconds beating (10 sec. pause) at room temperature at 6.5 m/sec on a



121 FastPrep-24 benchtop homogenizer (MP-Bio). Total DNA extractions were carried out using 80  
122  $\mu\text{L}$  (eq. 20 mg) of the homogenate and the Nucleospin 96 Tissue Kit (Macherey-Nagel)  
123 according to the manufacturer's protocol with minor adaptations (see detailed protocol in  
124 supplementary file 1). The remaining homogenate was flash frozen and stored at  $-80^{\circ}\text{C}$  for  
125 subsequent protein extractions. PCR mix was prepared with 1  $\mu\text{L}$  template DNA, 5  $\mu\text{L}$  GoTaq G2  
126 Flexi buffer, 0.15  $\mu\text{L}$  GoTaq polymerase enzyme (1 U/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  dNTP mix (each 10 mM),  
127 17.35  $\mu\text{L}$   $\text{H}_2\text{O}$ , and 0.5  $\mu\text{L}$  of forward and reverse primers specific to the *virB4* gene of the *V.*  
128 *tapetis* strain CECT4600<sup>T</sup> (final volume 25  $\mu\text{L}$ , adapted from Bidault et al. [28]). Initial  
129 denaturation was done at  $94^{\circ}\text{C}$  for 5 min, followed by 40 cycles of denaturation ( $94^{\circ}\text{C}$ ), annealing  
130 ( $54^{\circ}\text{C}$ ), and extension ( $72^{\circ}\text{C}$ ) for 30 seconds each, and a final extension step at  $72^{\circ}\text{C}$  for 3 min.  
131 PCR products were deposited on 1% agarose gel and electrophoresis was carried out at 110 V for  
132 45 min. In total DNA samples containing *V. tapetis* DNA, a 173 bp amplicon was then visible by  
133 fluorescence.

134

### 135 2.3. Total protein extraction and digestion

136 Based on both visual and molecular diagnoses, samples were assigned to one of four categories  
137 representative of the disease kinetics: BRD-/PCR- (0); BRD-/PCR+ (1); BRD+/PCR- (2);  
138 BRD+/PCR+ (3). Total proteins were extracted from three samples from category 0 and three  
139 samples from category 3 (presenting the same extent index of conchiolin deposit according to  
140 Paillard & Maes [29]), representing the extreme phenotypes hereafter referred to as "RES"  
141 (Category 0) and "DIS" (Category 3). Sample homogenates were defrosted on ice and 10  $\mu\text{L}$   
142 protease inhibitor mix (GE Healthcare) was added. After mixing by vortex, the samples were  
143 centrifuged at 15 000 x g for 10 min at  $4^{\circ}\text{C}$ . The supernatant was transferred to a clean tube and  
144 proteins were quantified according to the Bradford method [30]. Based on protein concentration,  
145 the volume necessary for 50  $\mu\text{g}$  of total proteins was transferred to a clean tube and volume was  
146 adjusted to 50  $\mu\text{L}$  with an ammonium bicarbonate (AmBic) solution (100 mM). Samples were  
147 reduced with 5  $\mu\text{L}$  dithiothreitol (10 mM) for 40 minutes at  $56^{\circ}\text{C}$ , then alkylated with 10  $\mu\text{L}$   
148 iodoacetamide (20 mM) for 30 min in the dark. Protein digestion was carried out at  $37^{\circ}\text{C}$   
149 overnight with 10  $\mu\text{L}$  trypsin buffer (0.1  $\mu\text{g}/\mu\text{L}$ ). After digestion, 5% formic acid was added and  
150 peptide samples were dehydrated using a SpeedVac<sup>TM</sup> concentrator.

151

152

153 *2.4. LC-MS/MS analyses*

154 Peptide quantification and identification was carried out through nano-LC MS/MS to allow for  
155 the comparison of the proteomic profiles of resistance to Brown Ring disease in the Manila clam.

156

157 *2.4.1. Sample Preparation for Mass Spectrometry Analysis*

158 For nano-LC fragmentation, peptide samples were first desalted and concentrated onto a  $\mu$ C18  
159 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute  
160 (Bruker Daltonics) ultra-high pressure nano flow chromatography system. Peptides were  
161 concentrated onto a C18 pepmap 100 (5 mm x 300  $\mu$ m i.d.) precolumn (Thermo Scientific) and  
162 separated at 50°C onto a Aurora reversed phase Reprosil column (25 cm x 75  $\mu$ m i.d.) packed  
163 with 1.6  $\mu$ m C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1%  
164 formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% ACN (v/v) (B). The nanoflow  
165 rate was set at 400 nl/min, and the gradient profile was as follows: from 2 to 15% B within 60  
166 min, followed by an increase to 25% B within 30 min and further to 37% within 10 min, followed  
167 by a washing step at 95% B and reequilibration.

168

169 *2.4.2. Mass Spectrometry Analysis*

170 MS experiments were carried out on a TIMS-TOF pro mass spectrometer (Bruker Daltonics) with  
171 a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). The system was  
172 calibrated each week and mass precision was greater than 1 ppm. A 1400 spray voltage with a  
173 capillary temperature of 180°C was typically employed for ionizing. MS spectra were acquired in  
174 the positive mode in the mass range from 100 to 1700 m/z. In the experiments described here, the  
175 mass spectrometer was operated in PASEF mode with exclusion of single charged peptides. A  
176 number of 10 PASEF MS/MS scans was performed during 1.25 seconds from charge range 2-5.

177

178 *2.4.3. Peptide Sequencing and Protein Precursor Identification*

179 The fragmentation pattern was used to determine the sequence of the peptide. Database searching  
180 was performed using the Peaks X software. A custom database was used, consisting in the  
181 translated sequences of loci from the digestive gland transcriptome of the Manila clam  
182 (unpublished data; a FASTA file containing loci sequences for significantly differentially

183 accumulated proteins can be found in supplementary file 3, with the corresponding annotations in  
184 supplementary file 2). The variable modifications allowed were as follows: C-Carbamidomethyl,  
185 K-acetylation, methionine oxidation, and Deamidation (NQ). “Trypsin” was selected as  
186 Semispecific. Mass accuracy was set to 30 ppm and 0.05 Da for MS and MS/MS mode  
187 respectively. Data were filtered according to a FDR of 0.5% and the elimination of protein  
188 redundancy on the basis of proteins being evidenced by the same set or a subset of peptides.

189

#### 190 *2.5. Data analysis*

191 Label-free quantitative data from Peaks X software were imported into Perseus in which  
192 statistical analyses were performed [31]. Data were log<sub>2</sub>-transformed and only proteins  
193 identified in every sample of at least one of the conditions tested were kept for further analysis.  
194 Data were then compared using a t-test between conditions “RES” and “DIS”, a threshold of  
195 significance of 0.05 was applied, below which proteins were considered as statistically  
196 differentially accumulated.

197

## 198 3. RESULTS AND DISCUSSION

199

## 200 3.1. Identification of RES vs DIS individuals following experimental infection

201 A major goal of this study was to compare the proteomic changes in *R. philippinarum*  
202 individuals, from a single population, showing contrasted susceptibilities to BRD. Overall, a low  
203 mortality of 2.4 % was observed during the four-week incubation period, as it often observed  
204 when *V. tapetis* is injected into the pallial fluids. Moreover, this low mortality occurred mostly  
205 on days 5-6, as was previously described by authors which suggested this is due to septicemia  
206 following accidental injections of *V. tapetis* in the tissues [32]. The highest mortality per group  
207 (5.5 %) occurred in group C1 (inoculated with FSSW), whereas group C2 (no treatment) and the  
208 experimental group (inoculated with *V. tapetis*) showed 1.8 % and 1.9 % total mortality,  
209 respectively, comparable to the results obtained in other BRD studies [13]. Shell length  
210 ( $12.37 \pm 0.14$  mm) and total weight ( $0.411 \pm 0.011$  g) were measured for all individuals, and dual  
211 diagnosis was carried out for 430 experimental clams. Dual diagnosis of the experimental clams  
212 showed that 59 % showed varying degrees of conchiolin deposits, and 42 % were PCR-positive  
213 for the strain-specific *virB4* 173 bp amplicon. Overall, the experimental population was relatively  
214 evenly distributed in the four categories (Table 1). Control groups C1 (n=66) and C2 (n=49)  
215 showed conchiolin deposits in 1.5 % and 2 % of individuals, respectively, and none of the control  
216 samples were positive for molecular diagnosis.

217 The four diagnostic categories in which the experimental clams were placed represent four stages  
218 in the infection process as it occurs within the extra pallial compartment, summarized in Figure 1.  
219 Clams from the two extreme phenotypes, i.e. category 0 (BRD-/PCR-) and category 3  
220 (BRD+/PCR+), were chosen for the following proteomic study.

221

222

## 223 3.2. Differential shotgun proteomics of resistant (RES) vs diseased (DIS) clams

224 In all, we could identify 2093 proteins, of which 2021 were present in at least 2 out of 3 samples  
225 in one or both condition(s) (termed “RES” for category 0/resistant and “DIS” for category  
226 3/diseased). Only proteins identified in both RES and DIS samples were retained for downstream  
227 analyses. Spectral counts were used to calculate the relative abundance of proteins. A Student T-  
228 test was used to identify proteins for which abundance was significantly modified in either of the

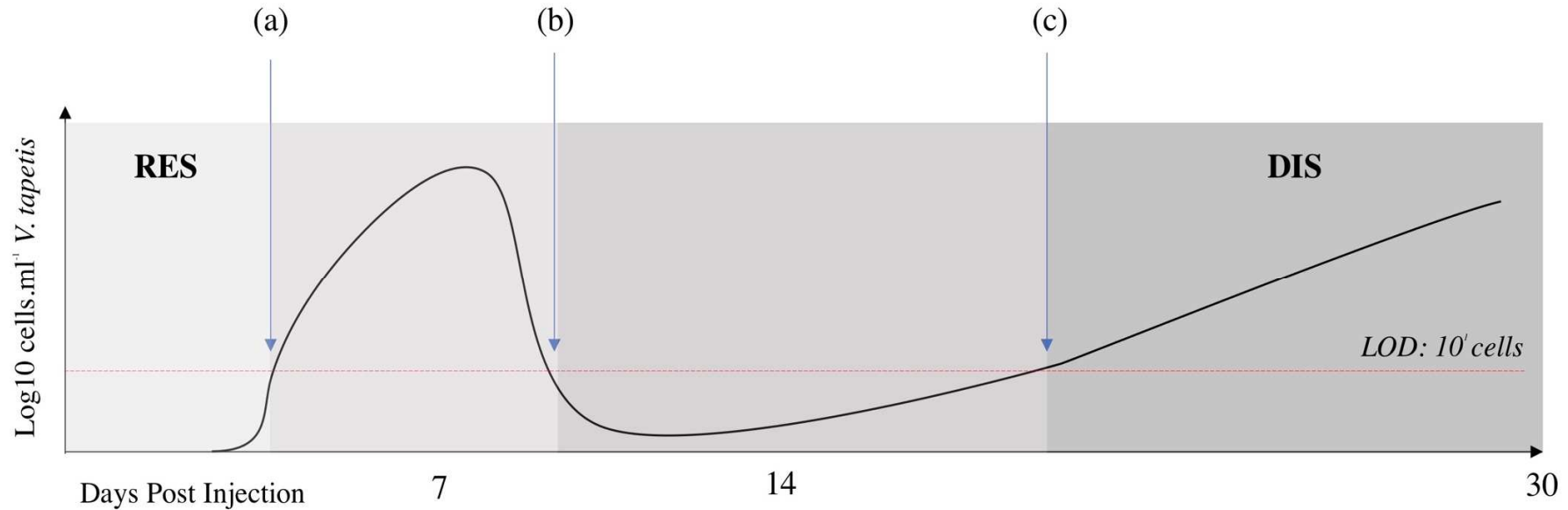
229 two categories, yielding a list of 102 proteins significantly more abundant in either RES or DIS  
 230 clams ( $p\text{-val} < 0.05$ ; a complete list of these proteins can be found in the supplementary file 2,  
 231 and a FASTA file containing the corresponding sequences of the loci can be found in  
 232 supplementary file 3). Of these, 49 proteins had a fold-change of at least 1.5: seventeen proteins  
 233 were accumulated in the RES group and thirty-two proteins in the DIS group, four of which could  
 234 not be characterized (*C. gigas* protein IDs: EKC23703, EKC34161, EKC41442, EKC37917).  
 235 These 49 proteins are presented in Figure 2 and their annotations are further detailed in Table 2;  
 236 they were functionally annotated by examining their associated COG categories, biological  
 237 process GO terms, and literature review, and discussed below according to their potential roles in  
 238 different aspects of Brown Ring disease, i.e. the “Immune response”, “Energy production” and  
 239 “Protein metabolism”.

240

Table 1: Dual diagnosis through visual inspection of the inner surface of both valves (BRD+ or BRD-) and PCR amplification of the virB4 gene region of 173 bp (PCR+ or PCR-) allows for distinction between 4 categories, ranging from CAT 0 (uninfected post-challenge, termed “RES”) to CAT 3 (infected according to both diagnostic methods, termed “DIS”). The 430 samples tested show that 24 % and 26 % of samples fall in CAT 0 and CAT 3, respectively. The link between these categories and the kinetics of infection in the extra pallial compartment with *Vibrio tapetis* is described in Figure 1.

#### DIAGNOSTIC CATEGORIES:

<i>Total experimental clams n= 430</i>	<b>BRD-</b>	<b>BRD+</b>
<b>PCR-</b>	<b>CAT 0</b> <i>n= 105; 24%</i>	<b>CAT 2</b> <i>n= 142; 33%</i>
<b>PCR+</b>	<b>CAT 1</b> <i>n= 70; 16%</i>	<b>CAT 3</b> <i>n= 113 ; 26%</i>



(ad. Bidault et al. 2015)

Figure 1: Schematic representation of the kinetics of Brown ring disease development during 30 days post injection (DPI) in a controlled challenge, adapted from Bidault et al. 2015, showing concentration of *V. tapetis* in extrapallial fluids ( $\text{cells.mL}^{-1}$ ) and limit of detection (LOD) at  $1 \times 10^1$  cells. "RES" clams are negative for both visual and molecular diagnosis. Bacteria enter the extrapallial compartment and become quantifiable at point (a), then proliferate rapidly (clams at this stage are termed "CAT 1" in our study), with highest concentrations generally observed around 7 DPI. The host then begins to trap the bacteria within the conchiolin deposits characteristic of Brown ring disease, thus leading to a decrease in the concentration of bacteria in the extrapallial compartment (clams at this stage are termed "CAT 2" in our study), represented by point (b). Clams can thus be positive for the visual diagnosis and negative for molecular diagnosis whilst the bacteria remain trapped against the inner surface of the shell, a process during which the host attempts to recalcify over the bacterial biofilm. In the case of "DIS" clams, conchiolin deposits are present but insufficient in limiting the pathogen, which will continue to proliferate (point (c), also termed "CAT 3" for this study) and reach high concentrations in the extrapallial compartment once more.

Disribution of significantly abundant proteins (FC > 1.5) in resistant ("RES") and diseased ("DIS") clams, by functional groups.

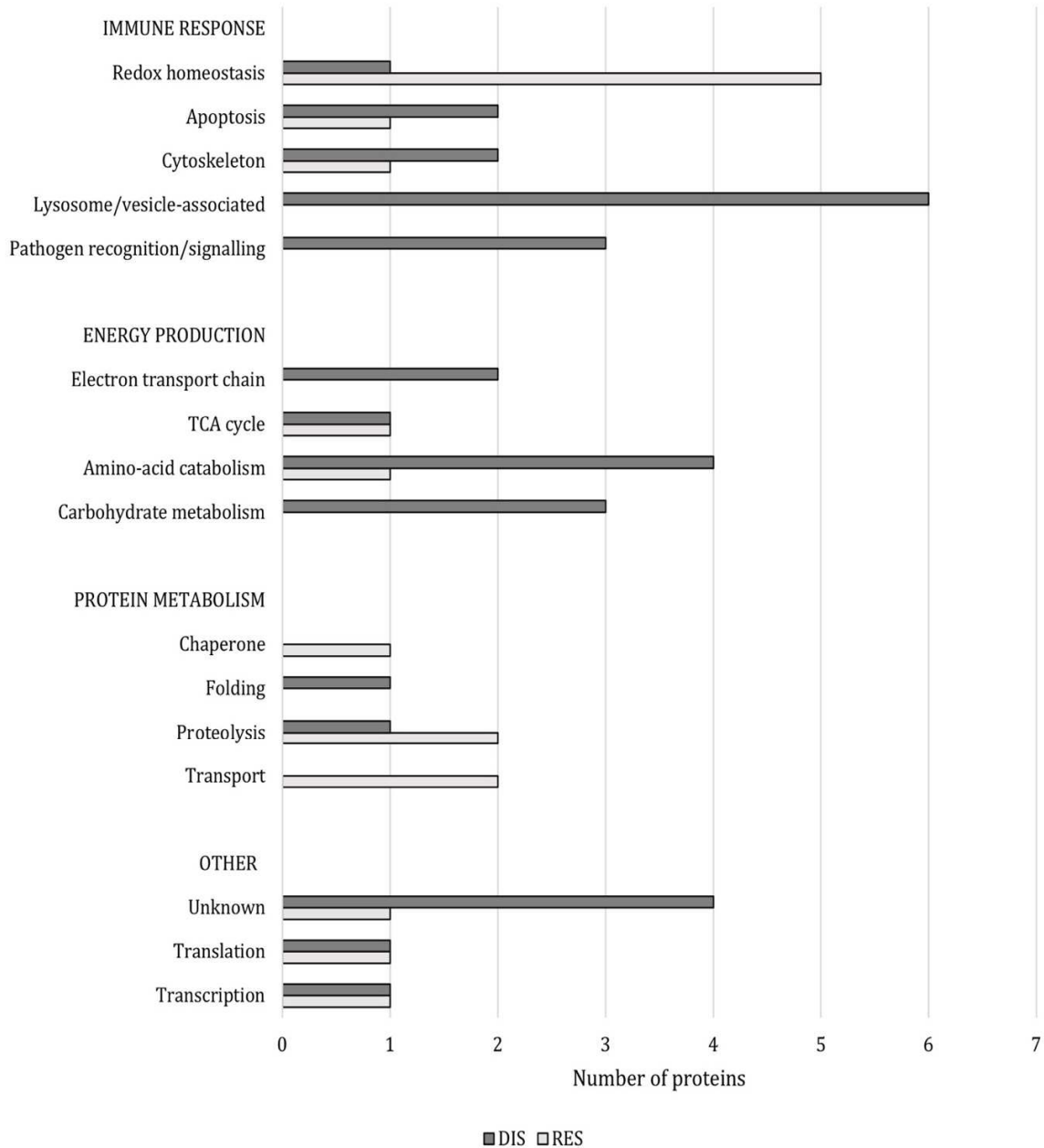


Figure 2: Number of proteins per functional group (i.e. "immune response", "energy production", "protein metabolism") that were more abundant in DIS clams (dark bars) and in RES clams (light bars).

Table 2: Proteins significantly abundant (p-val < 0.05) in DIS and RES clams, with fold-change > 1.5 ("group ratio"). Based on Clusters of Orthogous Groups (COG), Biological Process GO terms, and literature review regarding the roles of these proteins in the context of disease, the proteins are grouped into three main functional roles described in the discussion; "Immune response", "Energy production", and "Protein metabolism".

DIS Intensity	RES Intensity	Group Ratio <sup>†</sup>	Student's T-test p-value	Unique peptides	<i>R. philippinarum</i> locus ID	<i>C. gigas</i> protein ID	Protein name	COG	<i>C. gigas</i> gene description	Biological process GO term	Functional role discussed
1440	948	1.51:1.00	0.002	3	Locus 6868673	EKC40501	LGALS9	W	Galectin-9	GO:0006954 inflammatory response	Immune response - pathogen recognition
1250	421	2.97:1.00	0.041	1	Locus 5288240	EKC31577	BGBP	G	Beta-1,3-glucan-binding protein 1	GO:0002752 cell surface pattern recognition receptor signaling pathway	Immune response - pathogen recognition
384	244	1.57:1.00	0.026	1	Locus 4334179	EKC24393	C3	O	Complement C3	GO:0006955 immune response	Immune response - signalling
647	1770	2.73:1.00	0.005	3	Locus 615620	EKC23268	IQGAP1	Z	Ras GTPase-activating-like protein	GO:1903829 positive regulation of cellular protein localization	Immune response - cytoskeleton
1270	832	1.53:1.00	0.025	3	Locus 2955238	EKC27178	DCTN2	Z	Dynactin subunit 2	GO:0006888 endoplasmic reticulum to Golgi vesicle-mediated transport	Immune response - cytoskeleton
14900	4540	3.29:1.00	0.018	9	Locus 7235177	EKC29122	SCP	S	Sarcoplasmic calcium-binding protein	GO:0051480 regulation of cytosolic calcium ion concentration	Immune response - cytoskeleton
3200	2010	1.59:1.00	0.025	4	Locus 2215912	EKC27269	CPVL	O	Putative serine carboxypeptidase CPVL	GO:0051603 proteolysis involved in cellular protein catabolic process	Immune response - lysosomal
910	486	1.87:1.00	0.005	1	Locus 2384090	EKC25290	EPDR1	S	ependymin related 1	GO:0007160 cell-matrix adhesion	Immune response - lysosomal
903	446	2.03:1.00	0.045	1	Locus 2964637	EKC26355	OVCH1	O	Ovochymase-1	GO:0006508 proteolysis	Immune response - lysosomal
2660	531	5.02:1.00	0.029	2	Locus 3062641	ENSDARP00000139466*	PPT1	S	palmitoyl-protein thioesterase 1	GO:0007042 lysosomal lumen acidification	Immune response - lysosomal
680	374	1.82:1.00	0.014	1	Locus 665720	EKC31469	RAB43	U	Ras-related protein Rab-43	GO:0090382 phagosome maturation	Immune response - vesicles



1780	716	2.49:1.00	0.027	6	Locus 2853765	EKC32573	VPS35	U	Vacuolar protein sorting-associated protein 35	GO:0007040 lysosome organization	Immune response - vesicles
296	2550	8.61:1.00	0.003	3	Locus 6888709	EKC26119	ACE	C	Angiotensin-converting enzyme	GO:0001817 regulation of cytokine production	Immune response - redox
323	669	2.07:1.00	0.019	1	Locus 2057997	EKC35339	TXNDC5	O	Thioredoxin domain-containing protein 5	GO:0045454 cell redox homeostasis	Immune response - redox
4480	8140	1.82:1.00	0.045	3	Locus 355976	EKC36585	ACE	E	Angiotensin-converting enzyme	GO:0001817 regulation of cytokine production	Immune response - redox
152	245	1.61:1.00	0.048	1	Locus 4589062	EKC37227	ADH5	Q	Alcohol dehydrogenase class-3	GO:0051775 response to redox state	Immune response - redox
399	638	1.60:1.00	0.036	1	Locus 2688502	EKC36531	XDH	F	Xanthine dehydrogenase/oxidase	GO:2000379 positive regulation of reactive oxygen species metabolic process	Immune response - redox
2250	783	2.87:1.00	0.006	2	Locus 6590720	EKC20036	GPX3	O	Glutathione peroxidase	GO:0034599 cellular response to oxidative stress	Immune response - redox
448	753	1.68:1.00	0.003	2	Locus 3201607	EKC33267	SH3GLB1	T	Endophilin-B1	GO:0006915 apoptotic process	Immune response - apoptosis
1030	593	1.73:1.00	0.007	1	Locus 1202748	EKC29685	PPP3CC	T	Serine/threonine-protein phosphatase	GO:0035970 peptidyl-threonine dephosphorylation	Immune response - apoptosis
749	351	2.13:1.00	0.002	2	Locus 1979842	EKC21473	CECR1	F	Adenosine deaminase CECR1	GO:0006154 adenosine catabolic process	Immune response - apoptosis
3500	2120	1.65:1.00	0.036	1	Locus 1236277	EKC20480	AGL	G	Glycogen debranching enzyme	GO:0005980 glycogen catabolic process	Energy production – carbohydrate metabolism
2860	1720	1.67:1.00	0.007	3	Locus 8380444	EKC18570	PGM1	G	Phosphoglucomutase-1	GO:0006006 glucose metabolic process	Energy production - carbohydrate metabolism
11100	6930	1.60:1.00	0.037	7	Locus 381335	EKC27095	PCK2	C	Phosphoenolpyruvate carboxykinase [GTP]	GO:0006094 gluconeogenesis	Energy production - carbohydrate metabolism
1130	1770	1.57:1.00	0.031	2	Locus 2128190	EKC32958	AUH	I	Methylglutaconyl-CoA hydratase	GO:0006552 leucine catabolic process	Energy production - AAs
581	298	1.95:1.00	0.013	1	Locus 2982362	EKC42273	SLC1A1	E	Excitatory amino acid transporter 1	GO:0006537 glutamate biosynthetic process	Energy production - AAs

6150	1990	3.09:1.00	0.041	10	Locus 8587681	EKC33186	GPT2	E	Alanine aminotransferase 2	GO:0042851 L-alanine metabolic process	Energy production - AAs
21400	6060	3.54:1.00	0	19	Locus 2044774	EKC40669	GOT1	E	Aspartate aminotransferase, cytoplasmic	GO:0006107 oxaloacetate metabolic process	Energy production - AAs
4660	2820	1.65:1.00	0.034	1	Locus 7730822	EKC43060	PCCB	EI	Propionyl-CoA carboxylase beta chain	GO:0006552 leucine catabolic process	Energy production - AAs
9940	24000	2.41:1.00	0.038	8	Locus 4823168	EKC21276	PDHA1	C	Pyruvate dehydrogenase E1 subunit alpha	GO:0006086 acetyl-CoA biosynthetic process from pyruvate	Energy production - TCA
38700	24600	1.57:1.00	0.001	26	Locus 2348137	EKC25158	MDH1	C	Malate dehydrogenase	GO:0006099 tricarboxylic acid cycle	Energy production - TCA
1120	662	1.69:1.00	0.026	1	Locus 5269440	EKC19854	REBM	I	3-demethylubiquinone-9 3-methyltransferase	GO:0006744 ubiquinone biosynthetic process	Energy production - ETC
42700	24200	1.77:1.00	0.041	34	Locus 4313121	EKC39329	ATP5A1	C	ATP synthase subunit alpha	GO:0006754 ATP biosynthetic process	Energy production - ETC
1190	2290	1.92:1.00	0.049	3	Locus 1397283	EKC35325	TTN	T	Titin	GO:0007155 cell adhesion	Protein metabolism - transport
1940	2980	1.54:1.00	0.041	3	Locus 5145028	EKC29146	YWHAE	O	14-3-3 protein epsilon	GO:0035556 intracellular signal transduction	Protein metabolism - transport
1600	3590	2.24:1.00	0.034	10	Locus 3208485	EKC19309	CAND1	S	Cullin-associated NEDD8-dissociated protein 1	GO:0016567 protein ubiquitination	Protein metabolism - proteolysis
498	1630	3.27:1.00	0.044	2	Locus 4231109	EKC28114	PSMC1	O	26S proteasome non-ATPase regulatory subunit 7	GO:0000209 protein polyubiquitination	Protein metabolism - proteolysis
440	235	1.88:1.00	0.035	1	Locus 4900110	EKC29780	PSMD7	O	26S protease regulatory subunit 4	GO:0000209 protein polyubiquitination	Protein metabolism - proteolysis
4860	2510	1.94:1.00	0.012	4	Locus 2922613	EKC25378	FKBP14	O	FK506 binding protein 14	GO:0046716 muscle cell cellular homeostasis	Protein metabolism - protein folding
2550	4140	1.62:1.00	0.014	16	Locus 2939806	EKC25687	HSP90AB1	O	Heat shock protein HSP 90-alpha 1	GO:0050821 protein stabilization	Protein metabolism - chaperone

633	1200	1.90:1.00	0.048	2	Locus 2389731	EKC42074	PCBP3	A	Poly(RC)-binding protein 3	GO:0000122 negative regulation of transcription by RNA polymerase II	Other - transcription
1300	563	2.31:1.00	0.023	1	Locus 995640	EKC39351	PURA	K	Transcriptional activator protein Pur-alpha	GO:0006268 DNA unwinding involved in DNA replication	Other - transcription
1860	2940	1.58:1.00	0.003	1	Locus 453724	EKC20816	NARS	J	Asparaginyl-tRNA synthetase	GO:0006421 asparaginyl-tRNA aminoacylation	Other - translation
1300	752	1.73:1.00	0.024	2	Locus 454248	EKC31246	RPL27A	J	60S ribosomal protein L27a	GO:0006412 translation	Other - translation
818	1560	1.91:1.00	0.021	2	Locus 3238632	EKC23703	-	-	Uncharacterized - Calycin superfamily	---	Other - unknown
584	357	1.64:1.00	0.018	1	Locus 4959443	EKC41849	ABHD14A	S	Abhydrolase domain-containing protein 14A	GO:0006656 phosphatidylcholine biosynthetic process	Other - unknown
670	396	1.69:1.00	0.033	1	Locus 1004421	EKC34161	-	-	si:ch73-250a16.5	---	Other - unknown
3520	1910	1.85:1.00	0.042	6	Locus 6596965	EKC41442	-	-	Uncharacterized - SH3-like domain	---	Other - unknown
1060	350	3.03:1.00	0.028	3	Locus 4498780	EKC37917	-	-	Uncharacterized - Carbohydrate esterase 4 (CE4) family	---	Other - unknown
<p>† Ratios are presented as RES:DIS for those more abundant in RES clams, and as DIS:RES for those more abundant in DIS clams.</p> <p>* Danio rerio protein ID and protein description</p>											

### 3.3. IMMUNE RESPONSE-ASSOCIATED PROTEINS

#### 3.3.1. *Pathogen recognition and immune-pathway activation*

The ongoing infection process in clams from the DIS group is supported by the presence of a number of proteins whose primary functions are associated with pathogen recognition and the subsequent triggering of signaling pathways, such as Galectin-9 (Gal9), 1-3- $\beta$ -glucan-binding protein (BGBP), and Complement component C3 (C3). Proteins such as Galectins, which have previously been demonstrated as upregulated in the extra pallial fluids of *V. tapetis*-infected clams, and BGBP act as pattern recognition receptors (PRRs) by recognizing  $\beta$ -galactoside and lipopolysaccharide residues found on bacterial membranes [18,33,34]. This initiates the immune response by activating signaling pathways for chemotaxis, phagocytosis, and opsonization and induces antimicrobial peptide (AMP) synthesis through the prophenoloxidase and complement cascade systems [35,36]. Interestingly, selective breeding for parasite resistance in the Sydney rock oyster found that resistance was directly related to the loss of a specific form of phenoloxidase enzymes, supporting the presence of this particular enzyme as a marker of susceptibility [37,38]. The C3 protein identified in our dataset belongs to the complement cascade system, an essential component of the invertebrate immune response leading to the opsonization and lysis of pathogens [39–42]. Upregulation of proteins associated to the complement system have previously been described in *R. philippinarum* through several transcriptomic studies investigating response to disease, highlighting the importance of this pathway in host response to *Vibrio* pathogens [18,24]. The significantly high abundance of proteins specifically involved in pathogen recognition in the DIS group supports a strong acute response to the ongoing infection with *V. tapetis* in these animals.

Interestingly, the RES clam group showed high abundance of Ras GTPase-activating-like protein 1 (IQGAP1), a pathway-activating protein that is implicated in a number of immune-associated functions. Namely, during infection by microbial pathogens that target the host microtubule network, similar to the way in which *V. tapetis* inhibits pseudopod formation in the hemocytes of *R. philippinarum*, IQGAP1 has been shown to bind to and modulate the activity of proteins involved in bacterial invasion, ultimately interacting with the actin cytoskeleton [19,43].

274 3.3.2. *Pathogen-associated lysosomal activity*

275 Other proteins highly abundant in the DIS group are involved in immune response through  
276 antimicrobial and lysosomal activity. The putative serine carboxypeptidase (CPLV) and the  
277 serine protease Ovochymase (OVCH1) identified in the DIS group are known to have  
278 antibacterial activity and can be involved in proteolytic digestion of lysosomal components  
279 [44,45]. As lysosomes contain the hydrolytic enzymes necessary for degradation of cellular  
280 components as well as encapsulated pathogenic agents, it is also interesting to note the higher  
281 abundance of Ependymin-related protein 1 (EPDR1) in DIS samples, which has been suggested  
282 to function as a lysosomal activator protein, and Palmitoyl-protein thioesterase-1 (PPT1), which  
283 is associated with lysosomal degradation of proteins [46,47]. An uncharacterized protein in the  
284 DIS clam group belonging to the carbohydrate esterase 4 (CE4) family may also play a role in  
285 degrading phagocytosed bacteria as well as inhibiting biofilm formation, as certain enzymes of  
286 the CE4 family, whose main function is to de-acetylate polysaccharides, specifically degrade the  
287 essential peptidoglycan polymers of bacterial cell walls [48]. In addition, active intracellular  
288 membrane trafficking (ex. phagosomes) in DIS clams is suggested by the high abundance of  
289 proteins such as the Ras-related protein RAB43, which regulates vesicular movement following  
290 immune system activation by microbial infections, and a vacuolar-sorting protein VPS35, which  
291 directs transmembrane cargo proteins to the lysosomal degradation pathway [49,50].

292

293 3.3.3. *Cytoskeleton-associated immune response*

294 The cytoskeleton is a network of filaments that plays an essential role in certain aspects of  
295 immunity through cell structure maintenance, transport, phagocytosis, and communication  
296 between cellular components. As such, it is also known to be one of the targets of invading  
297 pathogens such as *Perkinsus olseni* and *V. tapetis* in the Manila clam [19,51,52]. Interestingly, a  
298 dynactin subunit (DCTN2) and a sarcoplasmic calcium-binding protein (SCP) were highly  
299 abundant in DIS clams, both of which play a role in cytoskeleton function. DCTN2 is part of a  
300 dynein/dynactin complex which coordinates the microtubule movement of vacuoles towards  
301 lysosomes and plays an important role in the biogenesis and transportation of pathogen-  
302 containing vacuoles in rabbit cells infected by obligate intracellular bacteria [53,54]. This may  
303 reflect the internalization of *V. tapetis* by hemocytes in the case of DIS clams, activating  
304 microtubule motors such as the dynein/dynactin complex in an attempt to fuse bacteria-

305 containing vacuoles with lysosomal membranes. SCPs, which are known to interact with the  
306 cytoskeleton by regulating the calcium balance, have been shown to be upregulated in Manila  
307 clam hemocytes in response to both parasitic and bacterial infections [18,22,51]. Bacteria of the  
308 genus *Vibrio* have been suggested to inhibit intracellular trafficking and the fusion of bacteria-  
309 containing phagosomes with lysosomes in order to avoid neutralization, a relatively common  
310 immune-evasion technique seen in a number of pathologies [55]. In this light, the elevated  
311 abundance of lysosome-associated proteins in clams of the DIS group may reflect an attempt to  
312 counter the bacteria's inhibitory processes.

313

#### 314 3.3.4. *Apoptotic processes*

315 Apoptosis of host immune cells during an infection can represent one of the ultimate defense  
316 strategies against invading pathogens, whereby the host cell is sacrificed so as to eliminate the  
317 internalized pathogenic agent [56]. Induction of apoptosis has been suggested to be one of the  
318 mechanisms of survival put in place by resistant oysters, *Ostrea edulis*, perhaps in response to  
319 anti-apoptotic mechanisms that are a known survival strategy for some intracellular pathogens,  
320 such as the protozoan parasite *Bonamia ostreae* [57]. Clams from the RES group in our study  
321 demonstrated higher abundances of Endophilin B1 (Bif-1), a protein associated with the  
322 formation of pores in the outer mitochondrial membrane that leads to apoptosis through the  
323 caspase pathway [57–59]. In contrast, DIS clams showed high abundance of adenosine  
324 deaminase (CECR1) a protein that, while primarily associated with the purine metabolism, also  
325 plays an important role in reducing the concentration of the toxic derivatives of adenosine and  
326 deoxyadenosine to protect cells from apoptosis [60,61]. A serine/threonine protein phosphatase  
327 PPP3CC, also more abundant in DIS clams, bears close resemblance with the protein phosphatase  
328 3 catalytic subunit beta, PPP3CB, which was recently suggested to promote cell-proliferation and  
329 may play an anti-apoptotic role in tumorous human kidney cells [62]. While apoptosis is a  
330 complex process that can benefit immune defense, anti-apoptosis is also a mechanism by which  
331 the host may maintain cellular functions and continue combatting infection, though seemingly at  
332 a cost given the relatively high abundance of proteins involved in energy metabolism in the DIS  
333 clam group (see part 3.4).

334

#### 335 3.3.5. *Redox homoeostasis*

336 Host production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a  
337 known mechanism of defense and pathogen neutralization, though this process requires a delicate  
338 balance as the accumulation of ROS/RNS can also lead to oxidative damage to the host [63].  
339 Glutathione peroxidase (GPX3), a member of the cellular antioxidant system, was more abundant  
340 in the DIS clam group. Interestingly, a previous studies on BRD-infected Manila clams reported a  
341 decrease in enzymatic activity of GPX at 7 dpi, though the study did not measure enzyme  
342 abundance nor activity at 30 dpi [21]. Oysters subjected to bacterial infection, however, have  
343 shown an upregulation of glutathione peroxidase gene expression in pathogen-challenged larvae,  
344 suggesting that GPX may be an indicator of oxidative stress that may have been occurring in DIS  
345 clams from our study as a result of the ongoing antibacterial processes discussed in part 3.3.2  
346 [21,64].

347 The RES clam group was characterized by a higher abundance of proteins more or less directly  
348 associated with ROS/RNS production and scavenging. Thioredoxin domain-containing protein 5  
349 (TXNDC5) is a member of the protein disulfide isomerase family whose expression has been  
350 associated with oxidative stress and cellular pathology [65,66]. At least two shorter-term (max 7  
351 days) studies have investigated the role of thioredoxin in response to *V. tapetis*, pointing towards  
352 an increase in activity of thioredoxin in *V. tapetis*-challenged clams compared to controls or to  
353 resistant clams, thus the higher abundance of thioredoxin observed in RES clams of this study  
354 may reflect a possible reversal of this mechanism in the case of long-term resistance (30 days)  
355 [21,67]. Similarly, xanthine oxidoreductase (XOR), an enzyme implicated in the purine  
356 metabolism, has previously been associated with a number of innate immunity processes  
357 including redox homeostasis through the production of ROS and RNS by xanthine oxidase (XO),  
358 as well as indirect free radical sequestration by xanthine dehydrogenase (XD) through the  
359 synthesis the antioxidant uric acid [63,68–70]. RES clams also presented a significantly higher  
360 abundance of two Angiotensin-converting enzymes (ACE), which were recently demonstrated to  
361 induce superoxide production and thereby enhance bactericidal activity in mouse neutrophils  
362 [71], and class-III alcohol dehydrogenase (ADH3). ADH3 uses the reducing power glutathione to  
363 eliminate the organic compounds formaldehyde and S-nitrosothiols (SNO), ultimately limiting  
364 their decomposition into the RNS nitric oxide [72,73]. Nitric oxide is a known toxic agent  
365 produced by immune cells of invertebrates for pathogen defense, and NO production by *R.*  
366 *philippinarum* in response to *V. tapetis* has been correlated to hemocyte rounding and pseudopod

367 loss [74], highlighting the possible importance of NO-sequestration by proteins such as ADH3 in  
368 order to counter the negative effects of this compound on the host.

369 These findings support previous hypotheses suggesting that ROS/RNS production by hemocytes  
370 may represent an alternative anti-bacterial response against pathogens such as *V. tapetis*, which is  
371 known to evade host immune responses such as phagocytosis by inhibiting pseudopod formation  
372 [74,75]. In addition, the higher abundance of the redox homeostasis-associated enzymes RES  
373 clams supports findings from previous studies indicating that resistance may be associated with a  
374 greater ability to balance ROS/RNS production and scavenging, allowing them to rapidly  
375 neutralize pathogens before they are able to colonize the host while simultaneously protecting  
376 host cells from oxidative damage, a dual process previously suggested using enzyme activity  
377 assays in the Manila clam exposed to *V. tapetis* [21].

378

### 379 3.4. PROCESSES ASSOCIATED WITH ENERGY PRODUCTION

380

381 Proteins potentially associated with energy production through carbohydrate and amino acid  
382 catabolism represent the second largest group in our dataset, most of which (10 out of 12) are  
383 more abundant in the DIS clam group. This section groups together the proteins implicated in the  
384 degradation of glycogen and amino acids that generate essential metabolites for the tricarboxylic  
385 acid (TCA) cycle, leading to the production of high-energy electron donors such as NADH which  
386 can integrate the electron transport chain (Figure 3).

387

#### 388 3.4.1. *Amino acid degradation for energy production*

389 Propionyl-CoA carboxylase beta chain (PCCB) and Methylglutaconyl-CoA hydratase (AUH),  
390 enzymes involved in the degradation of branched-chain amino acids (BCAAs) such as leucine,  
391 were significantly more abundant in DIS and RES clams, respectively. Demand for BCAAs as  
392 energy metabolites or for the synthesis of immune-related molecules has been demonstrated to  
393 increase during disease [76,77]. Decreased levels of a number of other amino acids during *Vibrio*  
394 infection in mussels have also been reported, suggesting that the significantly higher abundance  
395 of Alanine aminotransferase (GPT2), Aspartate aminotransferase (GOT1), and Excitatory amino-  
396 acid transporter-1 (SLC1A1) in DIS clams may also be associated with the degradation and/or



397 conversion of amino acids into metabolites such as pyruvate, oxaloacetate, and  $\alpha$ -ketoglutarate  
398 for the TCA cycle [78–81].

399

#### 400 3.4.2. *Carbohydrate metabolism and TCA cycle*

401 Our dataset showed a high abundance of proteins linking glycolysis with the TCA cycle for the  
402 aerobic production of ATP such as Pyruvate dehydrogenase (PDH) E1 subunit (alpha type III) in  
403 RES clams, as well as Glycogen debranching enzyme (AGL) and Phosphoglucomutase-1  
404 (PGM1) in DIS clams, which may reflect the degradation of carbohydrates for the production of  
405 pyruvate, namely in the DIS clam group [82]. This group is also characterized by a greater  
406 number of proteins associated with various aspects of the immune response, a process known to  
407 be energetically demanding [83]. More importantly, weight loss, decreased glycogen reserves,  
408 and condition index, which are indicative of energy imbalance and poor health, have previously  
409 been attributed to the negative impact of BRD on energy balance in the Manila clam [8,84,85].  
410 Malate dehydrogenase (MDH1), which participates in the TCA cycle by oxidizing malate to form  
411 oxaloacetate, was also more abundant in DIS clams, supporting the hypothesis of increased  
412 carbohydrate degradation for energy production. Our dataset also indicated a significantly higher  
413 abundance of the mitochondrial Phosphoenolpyruvate carboxykinase (PEPCK-M) in the DIS  
414 group, which is generally associated with catalyzing the irreversible conversion of oxaloacetate to  
415 phosphoenolpyruvate (PEP) for gluconeogenesis. While this appears to contradict the hypothesis  
416 of glycogen and glucose degradation, recent studies have demonstrated that overexpression of  
417 PEPCK-M (as opposed to the cytosolic form PEPCK-C) may play a role in antiviral immunity in  
418 insects, and the accumulation of PEP in infected ticks was suggested to be an antibacterial  
419 mechanism against the bacterial pathogen *Anaplasma phagocytophilum* [86,87].

420

#### 421 3.4.3. *Electron transport chain*

422 The end products of the TCA cycle ultimately convey their electrons to the electron transport  
423 chain (ETC), composed of molecules within the inner membrane of the mitochondria, including  
424 the essential proteins ubiquinone (coenzyme Q10) and ATP synthase. In the DIS group, the high  
425 abundance of 3-demethylubiquinone-9 3-O-methyltransferase, which participates in the final step  
426 of ubiquinone synthesis, suggests that there is a high demand for electron acceptor molecules  
427 which increase the proton gradient in the inter membrane space of the mitochondria [88].

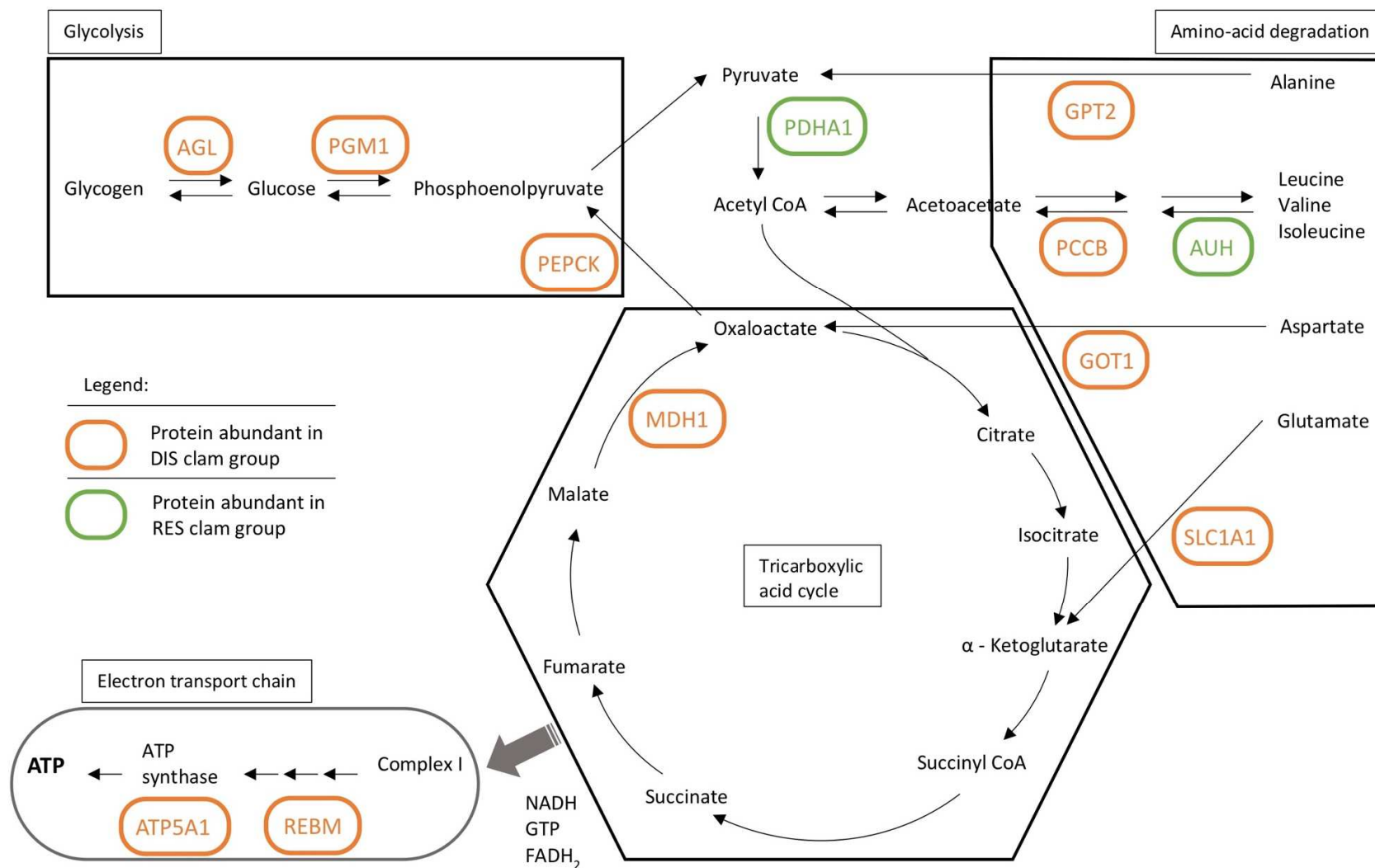


Figure 3: Schematic representation of energy production and the carbohydrate and amino acid metabolisms, including glycolysis, the TCA cycle, amino-acid degradation pathways, and the electron transport chain. The processes in which highly abundant proteins in DIS (orange) and RES (green) clams are implicated are annotated with the abbreviated protein name.

Glycogen debranching enzyme (AGL), Phosphoglucomutase-1 (PGM1), and Phosphoenolpyruvate carboxykinase (PEPCK) are associated to the carbohydrate metabolism (both glycolysis and gluconeogenesis). Alanine aminotransferase (GPT2), Propionyl-CoA carboxylase beta chain (PCCB), Methylglutaconyl-CoA hydratase (AUH), Aspartate aminotransferase (GOT1), and Excitatory amino-acid transporter-1 (SLC1A1) all participate in the degradation of amino acids that can play a role in replenishing metabolites of the TCA cycle. Malate dehydrogenase 1 (MDH1) is an essential enzyme of the TCA cycle, the high-energy products of which are then shuttles to the electron transport chain where proteins such as 3-demethylubiquinone-9 3-methyltransferase (REBM) and ATP synthase subunit alpha (ATP5A1) participate in the production of ATP.

429 Overall, proteins associated with the energy metabolism, including ATP synthase subunits and ETC-  
430 associated proteins, as well as proteins associated with energy production from both amino acids and  
431 sugars, are more abundant in the DIS group than in the RES group, reflecting a high energy demand  
432 likely as a result of the active immune response described previously.

433

### 434 3.5. PROTEIN METABOLISM

435 Seven highly abundant proteins in the dataset were associated with various aspects of protein  
436 metabolism, namely proteolysis, transport, and chaperones.

#### 437 3.5.1. *Proteolysis*

438 Proteolytic processes are represented in our dataset by the proteins cullin-associated NEDD8-  
439 dissociated protein 1 (CAND1) and subunit 4 of the 26S proteasome complex (PSMC1) in the RES  
440 group, and by the 26S proteasome non-ATPase regulatory subunit 7 (PSMD7) in the DIS group (Table  
441 2). In eukaryotic cells, proteolysis, or the degradation of proteins and recycling of their components, is  
442 a process mediated by the conjugation of polyubiquitin chains to proteins which are then recognized by  
443 the 26S proteasome complex, a multi-subunit enzyme responsible for proteolysis [89]. CAND1 binds  
444 to unneddylated CUL1, one of the three major components of an E3 ubiquitin ligase playing an  
445 essential role in protein degradation by regulating ligase ubiquitination [90]. Interestingly, ubiquitin  
446 ligase complexes were found to be one of the targets of pathogenic bacteria during infection, whereby  
447 inhibiting factors produced by pathogens may be able to effectively bind to CUL1, preventing it from  
448 correctly forming the ligase complex [91]. PSMC1 coordinates substrate recruitment and translocation  
449 into the proteolytic chamber of the proteasome, and is essential for rapid proteolysis [92]. Similarly,  
450 PSMD7 is another component of the proteasome important in mediating the recognition of  
451 polyubiquitin chains and cleavage of ubiquitin from degraded proteins [93]. In a previous gene-  
452 expression study of *P. olseni*-infected Manila clams, proteasome subunits were found to be  
453 downregulated in diseased animals, indicating decreased proteolytic activity [41]. In that respect, the  
454 elevated abundance of CAND1 and PSMC1 in RES clams may indicate a trend towards ubiquitin  
455 tagging of damaged proteins and more active protein degradation in RES clams than in DIS clams.

456 High proteolytic activity in RES clams may be linked to the digestion of phagocytosed and  
457 neutralized bacteria, and may also reflect the presence of a pro-apoptotic protein in this group. In  
458 addition, it may be possible that RES clams were able to sustain the cellular functions necessary for  
459 effective elimination of the pathogen, after which proteins damaged by oxidative stress during the  
460 immune response are degraded.

461 3.5.2. *Transport, folding, and chaperone functions*

462 Our dataset also contained proteins associated with various aspects of protein synthesis, including  
463 protein transport (Table 2). Titin (TTN), a large structural protein thought to function as a scaffold  
464 protein, has been shown to interact with actin and filamin, proteins of the cytoskeleton implicated in the  
465 movement of cellular components and proteins [94]. In vertebrate striated muscle, TTN was  
466 specifically shown to recruit E3 ubiquitin-ligase [95], thus its high abundance in RES clams may reflect  
467 transport associated with proteolysis, consistent with the fact that this group also showed higher  
468 abundance of CAND1 and a proteasome subunit. Another protein implicated in protein transport is 14-  
469 3-3 epsilon (YWHAE), a binding protein suggested to play a role in protein transport to the secretory  
470 pathway, namely through interaction with GTPase-activating proteins such as IQGAP1 (see 3.3.3  
471 *Cytoskeleton-associated immune response*), also found highly abundant in RES clams [96].

472 DIS clams showed a high abundance of FK506 binding protein 14 (FKBP14), which is thought to  
473 accelerate protein folding as it belongs to a family of peptidyl-prolyl cis-trans isomerases that play a  
474 role in the folding of newly synthesized proteins [97]. The higher abundance of this protein in the DIS  
475 group may reflect an increase in the synthesis of immune-related proteins, which were also more  
476 abundant in DIS clams. Furthermore, it is also interesting to note that this family of proteins (also  
477 called immunophilins) has been shown to inhibit early establishment and intracellular infection by  
478 bacteria [98]. Though not directly implicated in protein metabolism, the DIS group also presented high  
479 abundance of an Abhydrolase domain-containing protein associated with biosynthesis of  
480 phosphatidylcholine, an essential class of membrane phospholipids, possibly reflecting an increase in  
481 membrane synthesis due to the internalization of bacteria in phagosomes [99].

482 Heat-shock protein 90 (HSP90), a chaperone protein that plays a crucial role in protecting protein  
483 structure in response to stress conditions, was highly abundant in RES clams. Due to its interaction  
484 with the major histocompatibility complex (MHC) and the antigen processing pathways in vertebrates  
485 [100], HSP90 has been suggested to play a role in the innate immune system response and resistance to  
486 infection in invertebrates [101]. As HSP synthesis is promoted by protein denaturation, the trend  
487 towards proteolytic activity in RES clams discussed in part 3.5.1 may be partially responsible for  
488 activating HSP synthesis. That said, the higher abundance of HSP90 in RES clams may also indicate  
489 that resistant clams have a lower threshold for protein denaturation and thus perhaps more rapidly  
490 activate the synthesis of protective chaperone proteins, granting them an advantage over DIS clams  
491 when it comes to cellular protection.

492

## 493 3.6. OTHER

494 The transcriptional activator protein Pur-alpha (PURA), a protein involved in controlling DNA  
495 replication and gene transcription processes [102,103], was highly abundant in the DIS group, while a  
496 Poly (rc)-binding protein 3 (PCBP3) associated with negative regulation of transcription [104] was  
497 highly abundant in the RES group [104].

498 Proteins associated with translation include RPL27A, a structural component of the 60S ribosome  
499 subunit whose upregulation has previously been reported in white-spot infected shrimp and in hypoxia-  
500 stressed oysters [105,106], and asparaginyl-tRNA synthetase (NARS), whose primary function in  
501 translation is to catalyze the attachment of asparagine to its corresponding tRNA.

502 Finally, four significantly abundant proteins were uncharacterized; three were highly abundant in the  
503 DIS group and one in the RES group.

504

## 505 4. CONCLUSION

506 Despite being one of the fastest-growing sectors of aquaculture worldwide, mollusk production  
507 continues to suffer significant losses due in part to the impact of infectious diseases. The study of  
508 proteomic profiles offers the possibility of better understanding the complex functional mechanisms at  
509 play during host response to disease, and may shed light on factors associated with resistance to  
510 disease.

511 The aim of the present study was to investigate the proteomic profiles of resistance to controlled  
512 infection with *Vibrio tapetis*, the etiological agent of Brown Ring Disease, in the Manila clam,  
513 *Ruditapes philippinarum*. The comparison of proteomic profiles of two extreme phenotypes (RES and  
514 DIS) observed in juvenile Manila clams shows a number of functional differences in highly abundant  
515 proteins implicated in the immune response-associated processes, energy production, and protein  
516 metabolism. Twice as many significantly abundant proteins associated with the immune response were  
517 accumulated in the DIS group compared to the RES group, reflecting the ongoing infection as  
518 established by the presence of both visual and molecular signs of disease. That said, the function of  
519 immune-associated proteins in the RES group was almost consistently associated with redox  
520 homeostasis, whereas in DIS group the abundant proteins were mostly involved in pathogen  
521 recognition, signaling, and neutralization. This may suggest that disease resistant clams are better  
522 equipped to manage ROS production and scavenging in order to simultaneously eliminate the pathogen  
523 and protect host cellular components from oxidative stress. Protein degradation as well as protection by  
524 chaperones were another process highly represented in resistant clams, with degradation possibly as a

525 result of successful elimination of the pathogen which may nonetheless have left a number of cellular  
526 components damaged by oxidative stress. The fact that only resistant clams showed significantly high  
527 abundance of a chaperone-associated protein suggests that this may be an important factor of resistance  
528 to disease. In contrast, diseased clams showed a higher abundance of proteins involved in protein  
529 synthesis and functional modifications, possibly in response to activation by the immune system in  
530 order to continue fighting infection. Both immune response and protein synthesis are energy  
531 demanding processes, which is further supported by the presence of proteins involved in glycolysis,  
532 TCA cycle, and the electron transport chain. Overall, the comparison of the proteomic profiles of  
533 resistant and sick clams suggests that redox homeostasis and maintenance of protein structure by  
534 chaperone proteins may play important and interrelated roles in resistance to infection by *Vibrio tapetis*  
535 in the Manila clam.

536

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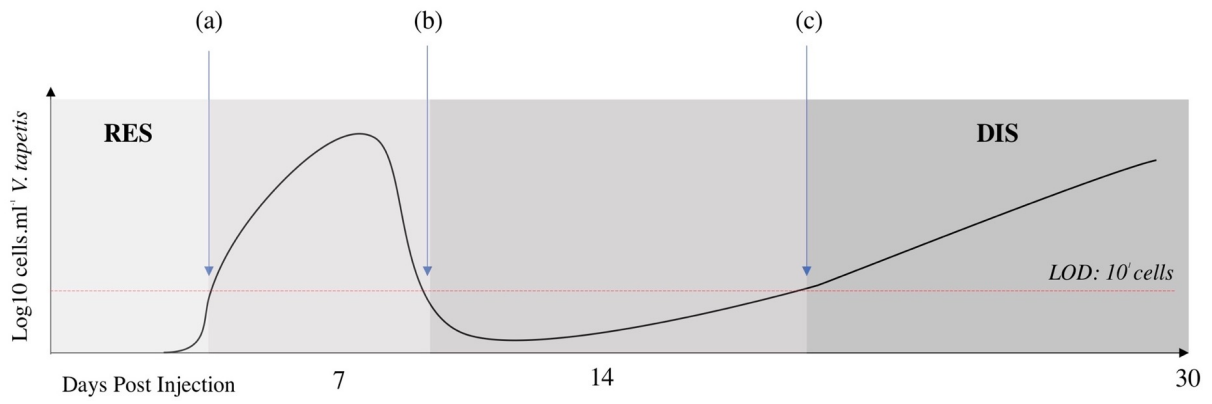
Figure 1: Schematic representation of the kinetics of Brown ring disease development during 30 days post injection (DPI) in a controlled challenge, adapted from Bidault et al. 2015, showing concentration of *V. tapetis* in extrapallial fluids ( $\text{cells.mL}^{-1}$ ) and limit of detection (LOD) at  $1 \times 10^1$  cells. "RES" clams are negative for both visual and molecular diagnosis. Bacteria enter the extrapallial compartment and become quantifiable at point (a), then proliferate rapidly (clams at this stage are termed "CAT 1" in our study), with highest concentrations generally observed around 7 DPI. The host then begins to trap the bacteria within the conchiolin deposits characteristic of Brown ring disease, thus leading to a decrease in the concentration of bacteria in the extrapallial compartment (clams at this stage are termed "CAT 2" in our study), represented by point (b). Clams can thus be positive for the visual diagnosis and negative for molecular diagnosis whilst the bacteria remain trapped against the inner surface of the shell, a process during which the host attempts to recalcify over the bacterial biofilm. In the case of "DIS" clams, conchiolin deposits are present but insufficient in limiting the pathogen, which will continue to proliferate (point (c), also termed "CAT 3" for this study) and reach high concentrations in the extrapallial compartment once more.



Figure 2: Number of proteins per functional group (i.e. “immune response”, “energy production”, “protein metabolism”) that were more abundant in DIS clams (dark bars) and in RES clams (light bars).

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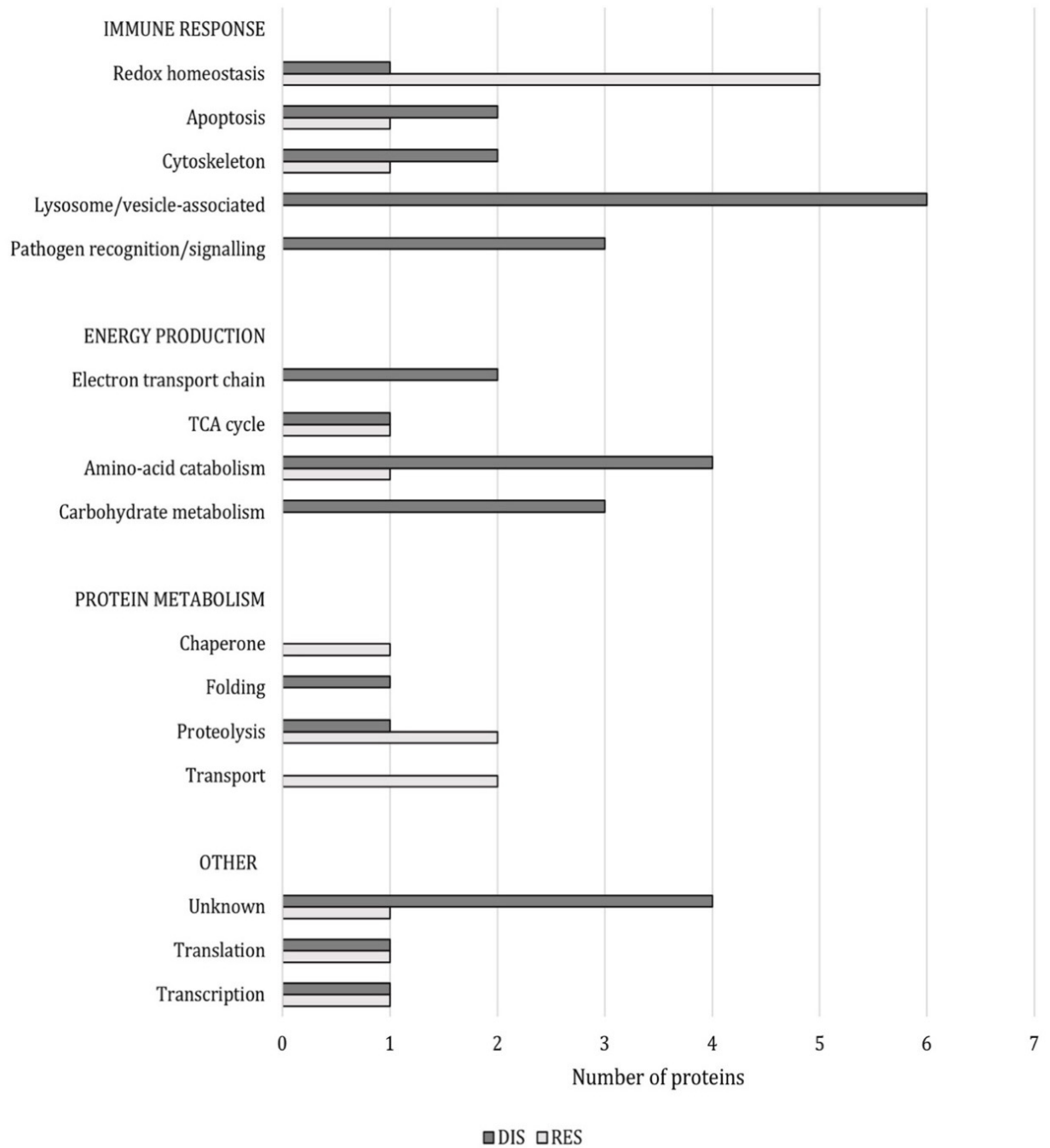
Figure 3: Schematic representation of energy production and the carbohydrate and amino acid metabolisms, including glycolysis, the TCA cycle, amino-acid degradation pathways, and the electron transport chain. The processes in which highly abundant proteins in DIS (orange) and RES (green) clams are implicated are annotated with the abbreviated protein name. Glycogen debranching enzyme (AGL), Phosphoglucomutase-1 (PGM1), and Phosphoenolpyruvate carboxykinase (PEPCK) are associated to the carbohydrate metabolism (both glycolysis and gluconeogenesis). Alanine aminotransferase (GPT2), Propionyl-CoA carboxylase beta chain (PCCB), Methylglutaconyl-CoA hydratase (AUH), Aspartate aminotransferase (GOT1), and Excitatory amino-acid transporter-1 (SLC1A1) all participate in the degradation of amino acids that can play a role in replenishing metabolites of the TCA cycle. Malate dehydrogenase 1 (MDH1) is an essential enzyme of the TCA cycle, the high-energy products of which are then shuttles to the electron transport chain where proteins such as 3-demethylubiquinone-9 3-methyltransferase (REBM) and ATP synthase subunit alpha (ATP5A1) participate in the production of ATP.

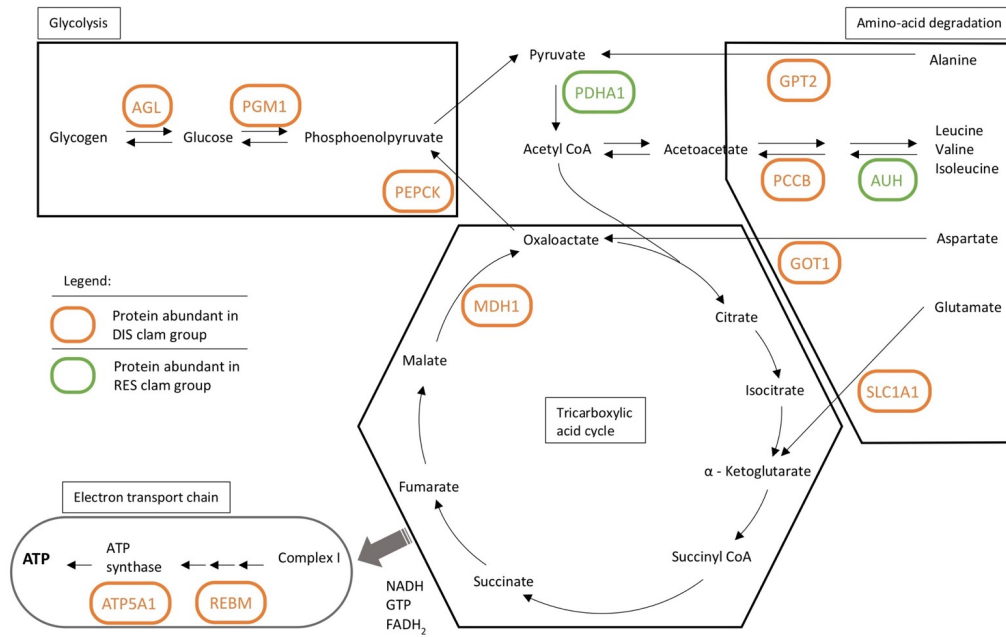


(ad. Bidault et al. 2015)

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Distribution of significantly abundant proteins (FC > 1.5) in resistant ("RES") and diseased ("DIS") clams, by functional groups.





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### Highlights

1. Diseased *Ruditapes philippinarum* show higher abundance of proteins associated with pathogen recognition and neutralization
2. Proteins associated with energy production metabolisms are more abundant in diseased clams
3. Healthy clams post-challenge showed higher abundance of proteins associated with redox homeostasis
4. Long-term resistance of *Ruditapes philippinarum* to infection with *Vibrio tapetis* may be linked to ability to balance redox response and maintain protein structure

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