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

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ABSTRACT

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

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

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

1. Introduction

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

In France, BRD continues to negatively impact production and prevalence can reach 80–100% [8] along the Northern Atlantic coast. While the severity of the disease and the virulence of its etiological agent are known to be largely dependent on a number of environmental factors, namely temperature and salinity, bivalves have a number of

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sophisticated stress and immune response mechanisms, as well as a highly specific innate immune system on which they rely to directly combat infection [9,10]. As marine bivalves lack an adaptive immune system, the innate genomic component of their immune system plays an essential role in mitigating the host response to pathogens.

Invertebrate innate immunity relies on a number of pathogen recognition factors that trigger signaling pathways involved in hemocyte recruitment, phagocytosis, and the production of a wide range of antimicrobial compounds for host defense. While resistance to infection in bivalves initially depends on the ability of mucosal interfaces to impede pathogen entrance into the host, circulating hemocytes from fluids (such as hemolymph and extra-pallial fluids) and bioactive molecules in the plasma are responsible for mediating the secondary host response through phagocytosis and direct bacterial neutralization by antimicrobial effectors [11,12]. Interestingly, clams have been shown to recover from BRD through shell repair processes, leading to the investigation of this resistant phenotype in several populations [13]. Previous gene expression and transcriptomic studies on the Manila clam have led to the assembly of a transcriptome and have shed light on several factors such as pathogen recognition and killing, modulation of hemocyte cytoskeleton, regulation of apoptosis, and bio-mineralization that are likely to play key roles in the innate immune response against bacterial infections [4–8,14–19]. The factors influencing virulence and highlighting the dynamics of the infection process leading to Brown Ring disease in the Manila clam are increasingly well described [8,20–23], and it has become clear that the interactions between host and pathogen during infection lead to a complex remodeling of the molecular framework of both organisms, highlighting the importance of understanding the changes in gene expression as well as those occurring on the proteomic level. In addition, several studies focusing on *Vibrio*-induced expression of immune-related genes in *R. philippinarum* suggest a tendency towards a downregulation of the inflammatory response and an upregulation of genes related to homeostasis in this species, insisting on the importance of investigating the molecular mechanisms at play to identify markers of resistance [24,25]. The growing number of genomic and transcriptomic resources available for this host species have unveiled particularly high levels of polymorphism, a factor that may mitigate the observed functional variability in the immune response to pathogens [26,27], though to date there remains a significant knowledge gap surrounding the functional response of the Manila clam to infection with *V. tapetis*, particularly in disease-resistant clams.

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

2. Materials and methods

2.1. Experimental design and sampling

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

2.2. Diagnostic methods

For visual diagnosis, shells were observed under a binocular magnifier to identify and quantify the extent index of Brown Ring disease according to the classification system described by Paillard & Maes (1994). For molecular diagnosis, DNA from whole-body tissue and fluid samples was analyzed. Briefly, the samples were homogenized in a volume of phosphate buffer saline (PBS; pH = 7) based on tissue weight, for a final concentration of 0.25 mg/µL tissue in the buffer. Ceramic beads were added to each sample and mechanical tissue disruption was done using 2 cycles of 20 s beating (10 s pause) at room temperature at 6.5 m/s on a FastPrep-24 benchtop homogenizer (MP-Bio). Total DNA extractions were carried out using 80 µL (eq. 20 mg) of the homogenate and the Nucleospin 96 Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol with minor adaptations (see detailed protocol in supplementary file 1). The remaining homogenate was flash frozen and stored at −80 °C for subsequent protein extractions. PCR mix was prepared with 1 µL template DNA, 5 µL GoTaq G2 Flexi buffer, 0.15 µL GoTaq polymerase enzyme (1 U/µL), 0.5 µL dNTP mix (each 10 mM), 17.35 µL H_20, and 0.5 µL of forward and reverse primers specific to the *virB4* gene of the *V. tapetis* strain CECT4600^T (final volume 25 µL, adapted from Bidault et al. [28]). Initial denaturation was done at 94 °C for 5 min, followed by 40 cycles of denaturation (94 °C), annealing (54 °C), and extension (72 °C) for 30 s each, and a final extension step at 72 °C for 3 min. PCR products were deposited on 1% agarose gel and electrophoresis was carried out at 110 V for 45 min. In total DNA samples containing *V. tapetis* DNA, a 173 bp amplicon was then visible by fluorescence.

2.3. Total protein extraction and digestion

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

2.4. LC-MS/MS analyses

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

2.4.1. Sample preparation for mass spectrometry analysis

For nano-LC fragmentation, peptide samples were first desalted and concentrated onto a μC18 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra-high pressure nano flow chromatography system. Peptides were concentrated onto a C18 pepmap 100 (5 mm × 300 μm i.d.) precolumn (Thermo Scientific) and separated at 50 °C onto a Aurora reversed phase Reprosil column (25 cm × 75 μm i.d.) packed with 1.6 μm C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% ACN (v/v) (B). The nanoflow rate was set at 400 nl/min, and the gradient profile was as follows: from 2 to 15% B within 60 min, followed by an increase to 25% B within 30 min and further to 37% within 10 min, followed by a washing step at 95% B and reequilibration.

2.4.2. Mass spectrometry analysis

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

2.4.3. Peptide sequencing and protein precursor identification

The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the Peaks X software. A custom database was used, consisting in the translated sequences of loci from the digestive gland transcriptome of the Manila clam (unpublished data; a FASTA file containing loci sequences for significantly differentially accumulated proteins can be sound in supplementary file 3, with the corresponding annotations in supplementary file 2). The variable modifications allowed were as follows: C-Carbamidomethyl, K-acetylation, methionine oxidation, and Deamidation (NQ). "Trypsin" was selected as Semispecific. Mass accuracy was set to 30 ppm and 0.05Da for MS and MS/MS mode respectively. Data were filtered according to a FDR of 0.5% and the elimination of protein redundancy on the basis of proteins being evidenced by the same set or a subset of peptides.

2.5. Data analysis

Label-free quantitative data from Peaks X software were imported into Perseus in which statistical analyses were performed [31]. Data were log2-transformed and only proteins identified in every sample of at least one of the conditions tested were kept for further analysis. Data were then compared using a *t*-test between conditions "RES" and "DIS", a threshold of significance of 0.05 was applied, below which proteins were considered as statistically differentially accumulated.

3. Results and discussion

3.1. Identification of RES vs DIS individuals following experimental infection

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

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 Fig. 1.

DIAGNOSTIC CATEGORIES:		
Total experimental clams n = 430	BRD-	BRD +
PCR-	**CAT 0** *n = 105*; 24%	**CAT 2** *n = 142*; 33%
PCR +	**CAT 1** *n = 70*; 16%	**CAT 3** *n = 113*; 26%

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

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

In all, we could identify 2093 proteins, of which 2021 were present in at least 2 out of 3 samples in one or both condition(s) (termed "RES" for category 0/resistant and "DIS" for category 3/diseased). Only proteins identified in both RES and DIS samples were retained for downstream analyses. Spectral counts were used to calculate the relative abundance of proteins. A Student T-test was used to identify proteins for which abundance was significantly modified in either of the two categories, yielding a list of 102 proteins significantly more abundant in either RES or DIS clams ($p\text{-}val < 0.05$; a complete list of these proteins can be found in the supplementary file 2, and a FASTA file containing the corresponding sequences of the loci can be found in supplementary file 3). Of these, 49 proteins had a fold-change of at least 1.5: seventeen proteins were accumulated in the RES group and thirty-two proteins in the DIS group, four of which could not be characterized (*C. gigas* protein IDs: EKC23703, EKC34161, EKC41442, EKC37917). These 49 proteins are presented in Fig. 2 and their annotations are further detailed in Table 2; they were functionally annotated by examining their associated COG categories, biological process GO terms, and literature review, and discussed below according to their potential roles in different aspects of Brown Ring disease, i.e. the "Immune response", "Energy production" and "Protein metabolism".

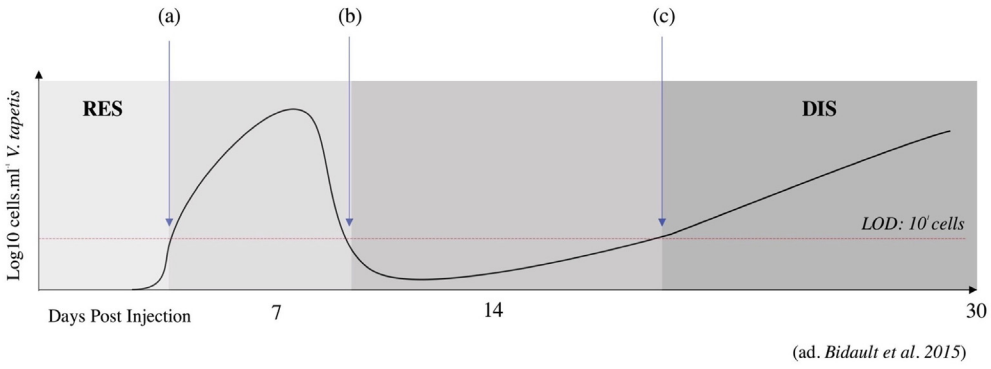

Fig. 1. Schematic representation of the kinetics of Brown Ring disease development during 30 days post injection (DPI) in a controlled challenge, adapted from Bidault et al., 2015, showing concentration of *V. tapetis* in extrapallial fluids (log10 cells.mL^{-1}) and limit of detection (LOD) at 1×10^1 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.

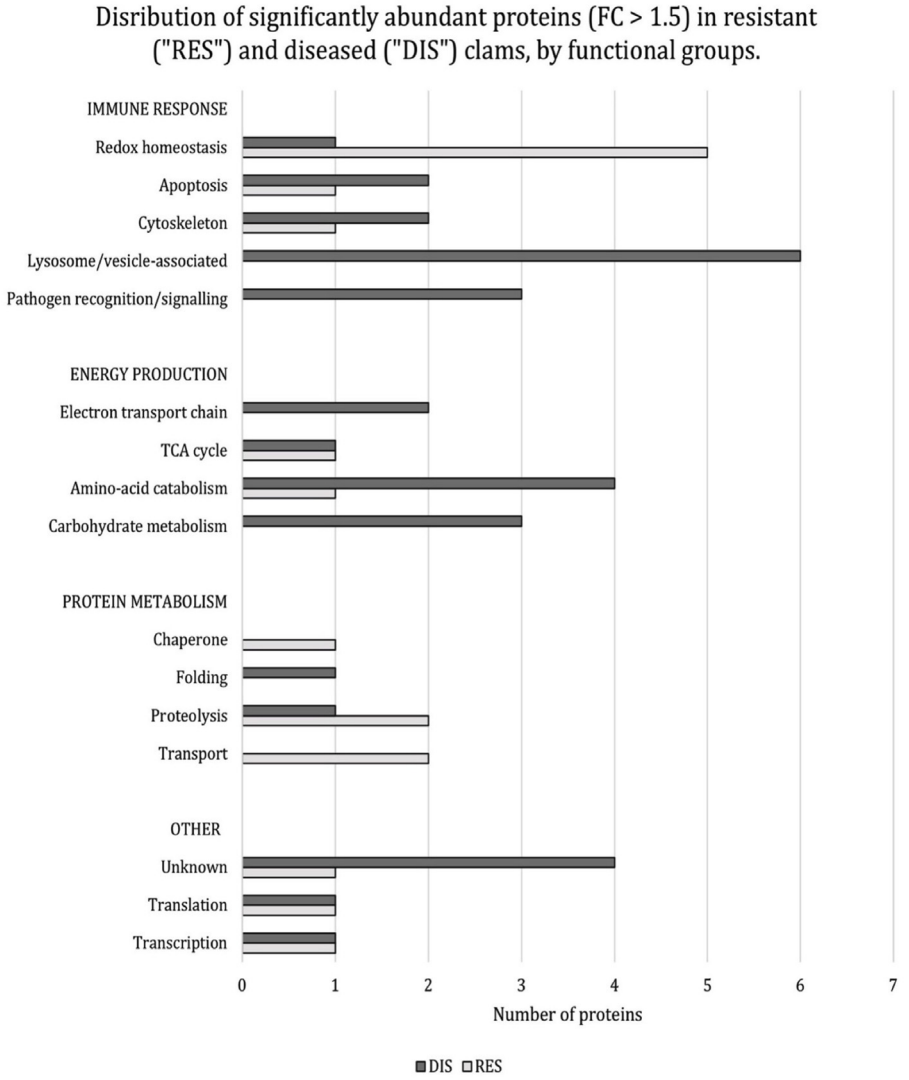

Fig. 2. Number of proteins per functional group (i.e. "immune response", "energy production", "protein metabolism") that were more abundant in DIS clams (dark bars) and in RES clams (light bars).

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†	Student's T-test p-value	Unique peptides	*R. philippinarum* locus ID	*C. gigas* protein ID	Protein name	COG	*C. gigas* 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

(*continued on next page*)

Table 2 (*continued*)

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:0045454cell 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

(*continued on next page*)

Table 2 (*continued*)

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

✝ Ratios are presented as RES:DIS for those more abundant in RES clams, and as DIS:RES for those more abundant in DIS clams.

* Danio rerio protein ID and protein description

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-β-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 β-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].

3.3.2. Pathogen-associated lysosomal activity

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

3.3.3. Cytoskeleton-associated immune response

The cytoskeleton is a network of filaments that plays an essential role in certain aspects of immunity through cell structure maintenance, transport, phagocytosis, and communication between cellular components. As such, it is also known to be one of the targets of invading pathogens such as *Perkinsus olseni* and *V. tapetis* in the Manila clam [19,51,52]. Interestingly, a dynactin subunit (DCTN2) and a sarcoplasmic calcium-binding protein (SCP) were highly abundant in DIS clams, both of which play a role in cytoskeleton function. DCTN2 is part of a dynein/dynactin complex which coordinates the microtubule movement of vacuoles towards lysosomes and plays an important role in the biogenesis and transportation of pathogen-containing vacuoles in rabbit cells infected by obligate intracellular bacteria [53,54]. This may reflect the internalization of *V. tapetis* by hemocytes in the case of DIS clams, activating microtubule motors such as the dynein/dynactin complex in an attempt to fuse bacteria-containing vacuoles with lysosomal membranes. SCPs, which are known to interact with the cytoskeleton by regulating the calcium balance, have been shown to be upregulated in Manila clam hemocytes in response to both parasitic and bacterial infections [18,22,51]. Bacteria of the genus *Vibrio* have been suggested to inhibit intracellular trafficking and the fusion of bacteria-containing phagosomes with lysosomes in order to avoid neutralization, a relatively common immune-evasion technique seen in a number of pathologies [55]. In this light, the elevated abundance of lysosome-associated proteins in clams of the DIS group may reflect an attempt to counter the bacteria's inhibitory processes.

3.3.4. Apoptotic processes

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

3.3.5. Redox homoeostasis

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

The RES clam group was characterized by a higher abundance of proteins more or less directly associated with ROS/RNS production and scavenging. Thioredoxin domain-containing protein 5 (TXNDC5) is a member of the protein disulfide isomerase family whose expression has been associated with oxidative stress and cellular pathology [65,66]. At least two shorter-term (max 7 days) studies have investigated the role of thioredoxin in response to *V. tapetis*, pointing towards an increase in activity of thioredoxin in *V. tapetis*-challenged clams compared to controls or to resistant clams, thus the higher abundance of thioredoxin observed in RES clams of this study may reflect a possible reversal of this mechanism in the case of long-term resistance (30 days) [21,67]. Similarly, xanthine oxidoreductase (XOR), an enzyme implicated in the purine metabolism, has previously been associated with a number of innate immunity processes including redox homeostasis through the production of ROS and RNS by xanthine oxidase (XO), as well as

indirect free radical sequestration by xanthine dehydrogenase (XD) through the synthesis the antioxidant uric acid [63,68–70]. RES clams also presented a significantly higher abundance of two angiotensin-converting enzymes (ACE), which were recently demonstrated to induce superoxide production and thereby enhance bactericidal activity in mouse neutrophils [71], and class-III alcohol dehydrogenase (ADH3). ADH3 uses the reducing power glutathione to eliminate the organic compounds formaldehyde and S-nitrosothiols (SNO), ultimately limiting their decomposition into the RNS nitric oxide [72,73]. Nitric oxide is a known toxic agent produced by immune cells of invertebrates for pathogen defense, and NO production by *R. philippinarum* in response to *V. tapetis* has been correlated to hemocyte rounding and pseudopod loss [74], highlighting the possible importance of NO-sequestration by proteins such as ADH3 in order to counter the negative effects of this compound on the host.

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

3.4. Processes associated with energy production

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

3.4.1. Amino acid degradation for energy production

Propionyl-CoA carboxylase beta chain (PCCB) and methylglutaconyl-CoA hydratase (AUH), enzymes involved in the degradation of branched-chain amino acids (BCAAs) such as leucine, were significantly more abundant in DIS and RES clams, respectively. Demand for BCAAs as energy metabolites or for the synthesis of immune-related molecules has been demonstrated to increase during disease [76,77]. Decreased levels of a number of other amino acids during *Vibrio* infection in mussels have also been reported, suggesting that the significantly higher abundance of alanine aminotransferase (GPT2), aspartate aminotransferase (GOT1), and excitatory amino-acid transporter-1 (SLC1A1) in DIS clams may also be associated with the degradation and/or conversion of amino acids into metabolites such as pyruvate, oxaloacetate, and α-ketoglutarate for the TCA cycle [78–81].

3.4.2. Carbohydrate metabolism and TCA cycle

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

3.4.3. Electron transport chain

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

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

3.5. Protein metabolism

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

3.5.1. Proteolysis

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

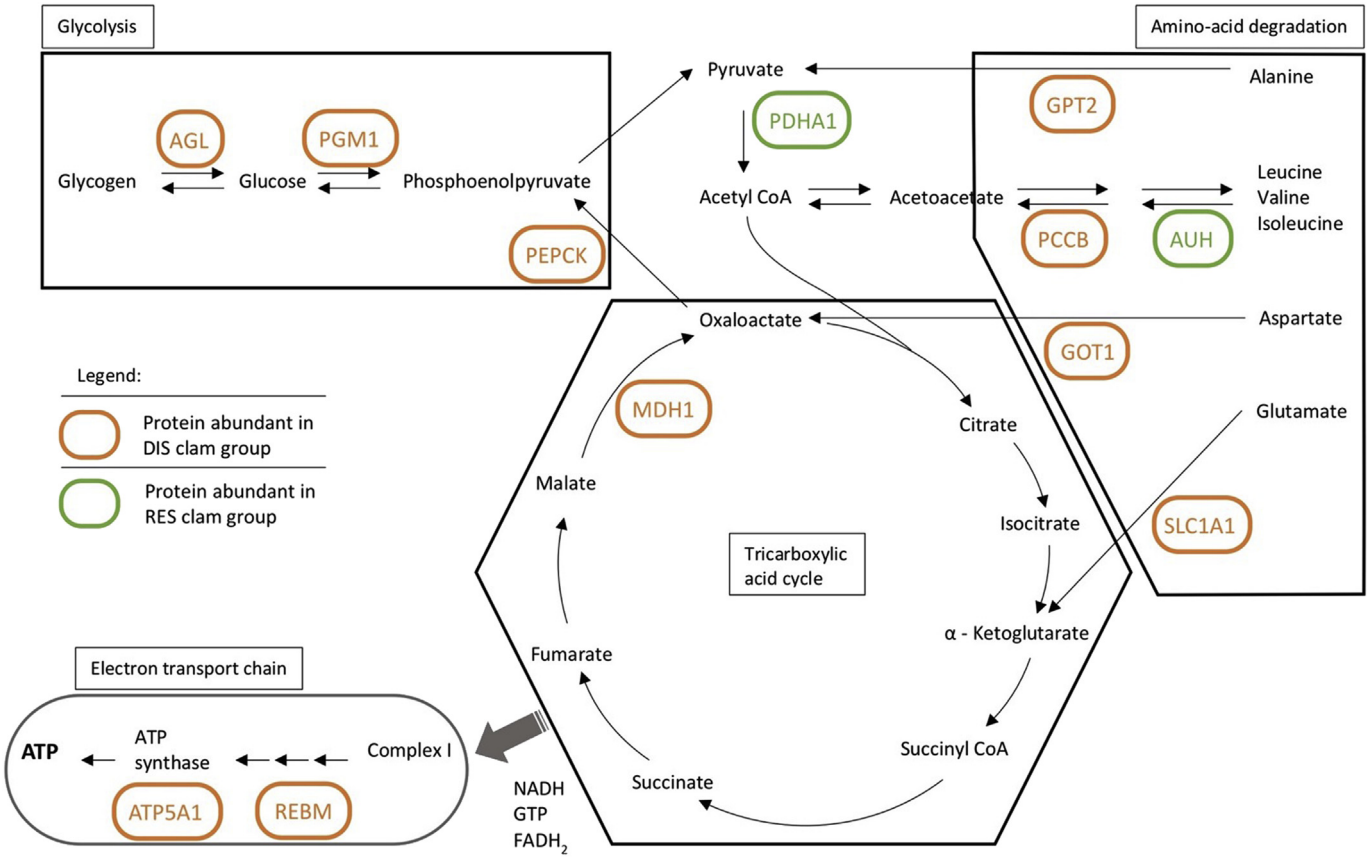

Fig. 3. Schematic representation of energy production and the carbohydrate and amino acid metabolisms, including glycolysis, the TCA cycle, amino-acid degradation pathways, and the electron transport chain. The processes in which highly abundant proteins in DIS (orange) and RES (green) clams are implicated 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

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

3.5.2. *Transport, folding, and chaperone functions*

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

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

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

when it comes to cellular protection.

3.6. Other

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

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

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

4. Conclusion

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

The aim of the present study was to investigate the proteomic profiles of resistance to controlled infection with *Vibrio tapetis*, the etiological agent of Brown Ring disease, in the Manila clam, *Ruditapes philippinarum*. The comparison of proteomic profiles of two extreme phenotypes (RES and DIS) observed in juvenile Manila clams shows a number of functional differences in highly abundant proteins implicated in the immune response-associated processes, energy production, and protein metabolism. Twice as many significantly abundant proteins associated with the immune response were accumulated in the DIS group compared to the RES group, reflecting the ongoing infection as established by the presence of both visual and molecular signs of disease. That said, the function of immune-associated proteins in the RES group was almost consistently associated with redox homeostasis, whereas in the DIS group the abundant proteins were mostly involved in pathogen recognition, signaling, and neutralization. This may suggest that disease resistant clams are better equipped to manage ROS production and scavenging in order to simultaneously eliminate the pathogen and protect host cellular components from oxidative stress. Protein degradation as well as protection by chaperones were also highly represented in resistant clams, with degradation possibly as a result of successful elimination of the pathogen which may nonetheless have left a number of cellular components damaged by oxidative stress. The fact that only resistant clams showed significantly high abundance of a chaperone-associated protein suggests that this may be an important factor of resistance to disease. In contrast, diseased clams showed a higher abundance of proteins involved in protein synthesis and functional modifications, possibly in response to activation by the immune system in order to continue fighting infection. Both immune response and protein synthesis are energy demanding processes, which is further supported by the presence of proteins involved in glycolysis, TCA cycle, and the electron transport chain. Overall, the comparison of the proteomic profiles of resistant and diseased clams suggests that redox homeostasis and maintenance of protein structure by chaperone proteins may play important and interrelated roles in resistance to infection by *Vibrio tapetis* in the Manila clam.

CRediT authorship contribution statement

M. Smits: Conceptualization, Methodology, Formal analysis, Writing - original draft. **S. Artigaud:** Formal analysis, Data curation, Validation, Writing - original draft. **B. Bernay:** Resources, Software, Formal analysis. **V. Pichereau:** Data curation, Validation, Supervision, Writing - review & editing. **L. Bargelloni:** Supervision, Funding acquisition, Resources. **C. Paillard:** Supervision, Funding acquisition, Project administration, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.02.002.

References

[1] FAO, The State of World Fisheries and Aquaculture 2018: Meeting the Sustainable Development Goals, (2018), https://doi.org/10.18356/8d6ea4b6-en.
[2] J. Flassch, Y. Leborgne, Introduction in Europe, from 1972 to 1980, of the Japanese manila clam, ICES Mar. Sci. Symp. 194 (1992) 92–96.
[3] C. Zannella, F. Mosca, F. Mariani, G. Franci, V. Folliero, M. Galdiero, P.G. Tiscar, M. Galdiero, Microbial diseases of bivalve mollusks: infections, immunology and antimicrobial defense, Mar. Drugs 15 (2017), https://doi.org/10.3390/md15060182.
[4] C. Paillard, P. Maes, R. Oubella, Brown ring disease in clams, Annu. Rev. Fish Dis. 4 (1994) 219–240, https://doi.org/10.1016/0959-8030(94)90030-2.
[5] C. Paillard, P. Maes, Etiologie de la maladie de l’anneau brun chez Tapes philippinarum: pathogénicité d’un Vibrio sp. Comptes Rendus l’Académie Des Sci. Série 3, Sci. La Vie. 310 (1990) 15–20.
[6] B. Allam, C. Paillard, M. Auffret, Alterations in hemolymph and extrapallial fluid parameters in the Manila Clam, Ruditapes philippinarum, challenged with the pathogen Vibrio tapetis, J. Invertebr. Pathol. 76 (2000) 63–69, https://doi.org/10.1006/jipa.2000.4940.
[7] C. Paillard, F. Le Roux, J.J. Borrego, Bacterial disease in marine bivalves, a review of recent studies: trends and evolution, Aquat. Living Resour. 17 (2004) 477–498, https://doi.org/10.1051/alr:2004054.
[8] C. Paillard, F. Jean, S.E. Ford, E.N. Powell, J.M. Klinck, E.E. Hofmann, J. Flye-Sainte-Marie, A theoretical individual-based model of Brown Ring Disease in Manila clams, Venerupis philippinarum, J. Sea Res. 91 (2014) 15–34, https://doi.org/10.1016/j.seares.2014.03.005.
[9] C. Paillard, B. Allam, R. Oubella, Effect of temperature on defense parameters in Manila clam Ruditapes philippinarum challenged with Vibrio tapetis, Dis. Aquat. Organ. 59 (2004) 249–262, https://doi.org/10.3354/dao059249.
[10] H.I. Reid, P. Soudant, C. Lambert, C. Paillard, T.H. Birkbeck, Salinity effects on immune parameters of Ruditapes philippinarum challenged with Vibrio tapetis, Dis. Aquat. Organ. 56 (2003) 249–258, https://doi.org/10.3354/dao056249.
[11] B. Allam, E. Pales Espinosa, Bivalve immunity and response to infections: are we looking at the right place? Fish Shellfish Immunol. 53 (2016) 4–12, https://doi.org/10.1016/j.fsi.2016.03.037.
[12] B. Allam, C. Paillard, Defense factors in clam extrapallial fluids, Dis. Aquat. Organ. 33 (1998) 123–128, https://doi.org/10.3354/dao033123.
[13] N. Trinkler, G. Sinquin, J. Querne, C. Paillard, Resistance to Brown Ring Disease in the Manila clam, Ruditapes philippinarum: a study of selected stocks showing a recovery process by shell repair, J. Invertebr. Pathol. 104 (2010) 8–16, https://doi.org/10.1016/j.jip.2009.12.007.
[14] F. Jeffroy, F. Brulle, C. Paillard, Differential expression of genes involved in immunity and biomineralization during Brown Ring Disease development and shell repair in the Manila clam, Ruditapes philippinarum, J. Invertebr. Pathol. 113 (2013) 129–136, https://doi.org/10.1016/J.JIP.2013.03.001.
[15] M. Milan, A. Coppe, R. Reinhardt, L.M. Cancela, R.B. Leite, C. Saavedra, C. Ciofi, G. Chelazzi, T. Patarnello, S. Bortoluzzi, L. Bargelloni, Transcriptome sequencing and microarray development for the Manila clam , Ruditapes philippinarum : genomic tools for environmental monitoring, BMC Genom. 12 (2011) 234, https://doi.org/10.1186/1471-2164-12-234.
[16] Y. Lee, W.D.N. Wickamarachchi, I. Whang, M. Oh, N. Umasuthan, M. De Zoysa, C. Oh, D.H. Kang, J. Lee, Immune response-related gene expression profile of a novel molluscan IκB protein member from Manila clam (Ruditapes philippinarum), Mol. Biol. Rep. 40 (2013) 1519–1527, https://doi.org/10.1007/s11033-

012-2196-5.
[17] U. Menike, K. Ariyasiri, J. Choi, Y. Lee, W.D.N. Wickramaarachchi, H.K.A. Premachandra, J. Lee, M. de Zoysa, Manila clam, Ruditapes philippinarum Cathepsin D : molecular analysis and immune response against brown ring disease causing Vibrio tapetis challenge, Korean J. Malacol. 29 (2013) 155–161, https://doi.org/10.9710/kjm.2013.29.2.155.
[18] B. Allam, E. Pales Espinosa, A. Tanguy, F. Jeffroy, C. Le Bris, C. Paillard, Transcriptional changes in manila clam (Ruditapes philippinarum) in response to Brown ring disease, Fish Shellfish Immunol. 41 (2014) 2–11, https://doi.org/10.1016/j.fsi.2014.05.022.
[19] F. Brulle, F. Jeffroy, S. Madec, J.L. Nicolas, C. Paillard, Transcriptomic analysis of Ruditapes philippinarum hemocytes reveals cytoskeleton disruption after in vitro Vibrio tapetis challenge, Dev. Comp. Immunol. 38 (2012) 368–376, https://doi.org/10.1016/j.dci.2012.03.003.
[20] G. Richard, C. Le Bris, F. Guérard, C. Lambert, C. Paillard, Immune responses of phenoloxidase and superoxide dismutase in the manila clam Venerupis philippinarum challenged with Vibrio tapetis - Part II: combined effect of temperature and two V.tapetis strains, Fish Shellfish Immunol. 44 (2015) 79–87, https://doi.org/10.1016/j.fsi.2014.12.039.
[21] G. Richard, F. Guérard, C. Corporeau, C. Lambert, C. Paillard, F. Pernet, Metabolic responses of clam Ruditapes philippinarum exposed to its pathogen Vibrio tapetis in relation to diet, Dev. Comp. Immunol. 60 (2016) 96–107, https://doi.org/10.1016/j.dci.2016.02.026.
[22] B. Marie, N. Trinkler, I. Zanella-Cleon, N. Guichard, M. Becchi, C. Paillard, F. Marin, Proteomic identification of novel proteins from the calcifying shell matrix of the manila clam venerupis philippinarum, Mar. Biotechnol. 13 (2011) 955–962, https://doi.org/10.1007/s10126-010-9357-0.
[23] C. Paillard, An ecological approach to understanding host-pathogen-environment interactions: the case of Brown ring disease in clams, Oysters Clams Cultiv. Habitat Threat. Ecol. Impact, Nova Science Publishers, Inc. (NOVA), 2016.
[24] R. Moreira, P. Balseiro, A. Romero, S. Dios, D. Posada, B. Novoa, A. Figueras, Gene expression analysis of clams Ruditapes philippinarum and Ruditapes decussatus following bacterial infection yields molecular insights into pathogen resistance and immunity, Dev. Comp. Immunol. 36 (2012) 140–149, https://doi.org/10.1016/j.dci.2011.06.012.
[25] R. Moreira, M. Milan, P. Balseiro, A. Romero, M. Babbucci, A. Figueras, L. Bargelloni, B. Novoa, Gene expression profile analysis of Manila clam (Ruditapes philippinarum) hemocytes after a Vibrio alginolyticus challenge using an immune-enriched oligo-microarray, BMC Genom. 15 (2014) 267, https://doi.org/10.1186/1471-2164-15-267.
[26] H. Nie, X. Yan, Z. Huo, L. Jiang, P. Chen, H. Liu, J. Ding, F. Yang, Construction of a high-density genetic map and quantitative trait locus mapping in the Manila clam Ruditapes philippinarum, Sci. Rep. 7 (2017) 1–9, https://doi.org/10.1038/s41598-017-00246-0.
[27] F. Ghiselli, A. Komissarov, L. Milani, J. Dunham, S. Breton, S. Nuzhdin, M. Passamonti, The Draft Genome of Ruditapes Philippinarum (The Manila Clam), a Promising Model System for Mitochondrial Biology, (2017), https://doi.org/10.7287/peerj.preprints.3096.
[28] A. Bidault, G. Richard, C. Le Bris, C. Paillard, Development of a Taqman real-time PCR assay for rapid detection and quantification of Vibrio tapetis in extrapallial fluids of clams, PeerJ 3 (2015) e1484, , https://doi.org/10.7717/peerj.1484.
[29] C. Paillard, P. Maes, Brown ring disease in the Manila clam Ruditapes philippinarum: establishment of a classification system, Dis. Aquat. Organ. 19 (1994) 137–146, https://doi.org/10.3354/dao019137.
[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[31] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M.Y. Hein, T. Geiger, M. Mann, J. Cox, The Perseus computational platform for comprehensive analysis of (prote) omics data, Nat. Methods 13 (2016) 731–740, https://doi.org/10.1038/nmeth.3901.
[32] B. Allam, S.E. Ford, Effects of the pathogenic Vibrio tapetis on defence factors of susceptible and non-susceptible bivalve species: I. Haemocyte changes following in vitro challenge, Fish Shellfish Immunol. 20 (2006) 374–383, https://doi.org/10.1016/j.fsi.2005.05.012.
[33] G.R. Vasta, C. Feng, M.A. Bianchet, T.R. Bachvaroff, S. Tasumi, Structural, functional, and evolutionary aspects of galectins in aquatic mollusks: from a sweet tooth to the Trojan horse, Fish Shellfish Immunol. 46 (2015) 94–106, https://doi.org/10.1016/j.fsi.2015.05.012.
[34] A. Chaosomboon, B. Phupet, O. Rattanaporn, P. Runsaeng, P. Utarabhand, Lipopolysaccharide- and β-1,3-glucan-binding protein from Fenneropenaeus merguiensis functions as a pattern recognition receptor with a broad specificity for diverse pathogens in the defense against microorganisms, Dev. Comp. Immunol. 67 (2017) 434–444, https://doi.org/10.1016/j.dci.2016.07.006.
[35] S.S. Jayaraj, R. Thiagarajan, M. Arumugam, P. Mullainadhan, Isolation, purification and characterization of β-1,3-glucan binding protein from the plasma of marine mussel Perna viridis, Fish Shellfish Immunol. 24 (2008) 715–725, https://doi.org/10.1016/j.fsi.2007.11.012.
[36] D. Ricklin, E.S. Reis, D.C. Mastellos, P. Gros, J.D. Lambris, Complement component C3 – the “Swiss Army Knife” of innate immunity and host defense, Immunol. Rev. 274 (2016) 33–58, https://doi.org/10.1111/imr.12500.
[37] D.A. Raftos, R. Kuchel, S. Aladaileh, D. Butt, Infectious microbial diseases and host defense responses in sydney rock oysters, Front. Microbiol. 5 (2014) 135, https://doi.org/10.3389/fmicb.2014.00135.
[38] B. Bezemer, D. Butt, J. Nell, R. Adlard, D. Raftos, Breeding for QX disease resistance negatively selects one form of the defensive enzyme, phenoloxidase, in Sydney rock oysters, Fish Shellfish Immunol. 20 (2006) 627–636, https://doi.org/10.1016/j.fsi.2005.08.007.
[39] H. Zhang, L. Song, C. Li, J. Zhao, H. Wang, L. Qiu, D. Ni, Y. Zhang, A novel C1q-domain-containing protein from Zhikong scallop Chlamys farreri with lipopolysaccharide binding activity, Fish Shellfish Immunol. 25 (2008) 281–289, https://doi.org/10.1016/j.fsi.2008.06.003.
[40] M. Gerdol, C. Manfrin, G. De Moro, A. Figueras, B. Novoa, P. Venier, A. Pallavicini, The C1q domain containing proteins of the Mediterranean mussel Mytilus galloprovincialis: a widespread and diverse family of immune-related molecules, Dev. Comp. Immunol. 35 (2011) 635–643, https://doi.org/10.1016/j.dci.2011.01.018.
[41] A. Romero, G. Forn-Cuní, R. Moreira, M. Milan, L. Bargelloni, A. Figueras, B. Novoa, An immune-enriched oligo-microarray analysis of gene expression in Manila clam (Venerupis philippinarum) haemocytes after a Perkinsus olseni challenge, Fish Shellfish Immunol. 43 (2015) 275–286, https://doi.org/10.1016/j.fsi.2014.12.029.
[42] J. Arivalagan, T. Yarra, B. Marie, V.A. Sleight, E. Duvernois-Berthet, M.S. Clark, A. Marie, S. Berland, Insights from the shell proteome: biomineralization to adaptation, Mol. Biol. Evol. 34 (2017) 66–77, https://doi.org/10.1093/molbev/msw219.
[43] H. Kim, C.D. White, D.B. Sacks, IQGAP1 in microbial pathogenesis: targeting the actin cytoskeleton, FEBS Lett. 585 (2011) 723–729, https://doi.org/10.1016/j.febslet.2011.01.041.
[44] J. Harris, N. Schwinn, J.A. Mahoney, H.-H.H. Lin, M. Shaw, C.J. Howard, R.P. da Silva, S. Gordon, A vitellogenic-like carboxypeptidase expressed by human macrophages is localized in endoplasmic reticulum and membrane ruffles, Int. J. Exp. Pathol. 87 (2006) 29–39, https://doi.org/10.1111/j.0959-9673.2006.00450.x.
[45] K. Gao, S. Zhang, Ovochymase in amphioxus Branchiostoma belcheri is an ovary-specific trypsin-like serine protease with an antibacterial activity, Dev. Comp. Immunol. 33 (2009) 1219–1228, https://doi.org/10.1016/j.dci.2009.07.002.
[46] Y. Wei, Z.J. Xiong, J. Li, C. Zou, C.W. Cairo, J.S. Klassen, G.G. Privé, Crystal structures of human lysosomal EPDR1 reveal homology with the superfamily of bacterial lipoprotein transporters, Commun. Biol. 2 (2019) 1–13, https://doi.org/10.1038/s42003-018-0262-9.
[47] B.A. Bannan, J. Van Etten, J.A. Kohler, Y. Tsoi, N.M. Hansen, S. Sigmon, E. Fowler, H. Buff, T.S. Williams, J.G. Ault, R.L. Glaser, C.A. Korey, The drosophila protein palmitoylome characterizing palmitoyl-thioesterases and DHHC palmitoyl-transferases, Fly 2 (2008) 198–214, https://doi.org/10.4161/fly.6621.
[48] S. Balomenou, S. Arnaouteli, D. Koutsioulis, V.E. Fadouloglou, V. Bouriotis, Polysaccharide deacetylases: new antibacterial drug targets, Front. Anti-infective Drug Discov, 2015, pp. 68–130, , https://doi.org/10.2174/9781681080826115040005.
[49] G. Pei, M. Bronietzki, M.G. Gutierrez, Immune regulation of Rab proteins expression and intracellular transport, J. Leukoc. Biol. 92 (2012) 41–50, https://doi.org/10.1189/jlb.0212076.
[50] R. Linhart, S.A. Wong, J. Cao, M. Tran, A. Huynh, C. Ardrey, J.M. Park, C. Hsu, S. Taha, R. Peterson, S. Shea, J. Kurian, K. Venderova, Vacuolar protein sorting 35 (Vps35) rescues locomotor deficits and shortened lifespan in Drosophila expressing a Parkinson's disease mutant of Leucine-rich repeat kinase 2 (LRRK2), Mol. Neurodegener. 9 (2014) 1–10, https://doi.org/10.1186/1750-1326-9-23.
[51] S. Fernández-Boo, A. Villalba, A. Cao, Protein expression profiling in haemocytes and plasma of the Manila clam Ruditapes philippinarum in response to infection with Perkinsus olseni, J. Fish. Dis. 39 (2016) 1369–1385, https://doi.org/10.1111/jfd.12470.
[52] A. Rahmani, E. Corre, G. Richard, A. Bidault, L. Oliveira, F. Thompson, C. Paillard, V. Pichereau, Transcriptomic analysis of clam extra pallial fluids reveals immunity and cytoskeleton alterations in the first week of brown ring disease development, Fish Shellfish Immunol. 91 (2019) 466, https://doi.org/10.1016/j.fsi.2019.04.275.
[53] R.M. Ortiz Flores, J.S. Distel, M.O. Aguilera, W. Berón, The Role of Microtubules and the Dynein/Dynactin Motor Complex of Host Cells in the Biogenesis of the Coxiella Burnetii-Containing Vacuole, (2019), https://doi.org/10.1371/journal.pone.0209820.
[54] S.R. Gill, T.A. Schroer, I. Szilak, E.R. Steuer, M.P. Sheetz, D.W. Cleveland, Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein, J. Cell Biol. 115 (1991) 1639–1650, https://doi.org/10.1083/jcb.115.6.1639.
[55] R.S. Flannagan, G. Cosío, S. Grinstein, Antimicrobial mechanisms of phagocytes and bacterial evasion strategies, Nat. Rev. Microbiol. 7 (2009) 355–366, https://doi.org/10.1038/nrmicro2128.
[56] A. Romero, B. Novoa, A. Figueras, The complexity of apoptotic cell death in mollusks: an update, Fish Shellfish Immunol. 46 (2015) 79–87, https://doi.org/10.1016/j.fsi.2015.03.038.
[57] B. Morga, T. Renault, N. Faury, I. Arzul, New insights in flat oyster Ostrea edulis resistance against the parasite Bonamia ostreae, Fish Shellfish Immunol. 32 (2012) 958–968, https://doi.org/10.1016/j.fsi.2012.01.026.
[58] J. Pawlowski, A.S. Kraft, Bax-induced apoptotic cell death, Proc. Natl. Acad. Sci. Unit. States Am. 97 (2002) 529–531, https://doi.org/10.1073/pnas.97.2.529.
[59] Y. Takahashi, M. Karbowski, H. Yamaguchi, A. Kazi, J. Wu, S.M. Sebti, R.J. Youle, H.-G. Wang, Loss of Bif-1 suppresses Bax/Bak conformational change and mitochondrial apoptosis, Mol. Cell Biol. 25 (2005) 9369–9382, https://doi.org/10.1128/mcb.25.21.9369-9382.2005.
[60] H. Mutsuzaki, H. Nakajima, Y. Wadano, S. Furuhata, M. Sakane, Influence of knee immobilization on chondrocyte apoptosis and histological features of the anterior cruciate ligament insertion and articular cartilage in rabbits, Int. J. Mol. Sci. 18 (2017), https://doi.org/10.3390/ijms18020253.
[61] G.H. Semerjyan, H.H. Semerjyan, A.H. Trchounian, Effects of Mg2+, Cd2+,

Cu2+ low concentrations and immobilization stress on the activity of adenosine deaminase in different organs of rabbits, Proc. Yerevan State Univ. Chem. Biol. 52 (2018) 122–127.
[62] L. Chen, Q. He, Y. Liu, Y. Wu, D. Ni, J. Liu, Y. Hu, Y. Gu, Y. Xie, Q. Zhou, Q. Li, PPP3CB inhibits migration of G401 cells via regulating epithelial-to-mesenchymal transition and promotes G401 cells growth, Int. J. Mol. Sci. 20 (2019) 275, https://doi.org/10.3390/ijms20020275.
[63] R.S. Anderson, Reactive oxygen species and antimicrobial defenses of invertebrates: a bivalve model, Adv. Exp. Med. Biol. (2001) 131–139, https://doi.org/10.1007/978-1-4615-1291-2_12.
[64] B. Genard, P. Miner, J.L. Nicolas, D. Moraga, P. Boudry, F. Pernet, R. Tremblay, Integrative study of physiological changes associated with bacterial infection in Pacific oyster larvae, PloS One 8 (2013), https://doi.org/10.1371/journal.pone.0064534.
[65] H.A. Chawsheen, Q. Ying, H. Jiang, Q. Wei, A critical role of the thioredoxin domain containing protein 5 (TXNDC5) in redox homeostasis and cancer development, Genes Dis. 5 (2018) 312–322, https://doi.org/10.1016/j.gendis.2018.09.003.
[66] E. Horna-Terrón, A. Pradilla-Dieste, C. Sánchez-de-Diego, J. Osada, TXNDC5, a newly discovered disulfide isomerase with a key role in cell physiology and pathology, Int. J. Mol. Sci. 15 (2014) 23501–23518, https://doi.org/10.3390/ijms151223501.
[67] N. Umasuthan, K. Saranya Revathy, Y. Lee, I. Whang, J. Lee, Mitochondrial thioredoxin-2 from Manila clam (Ruditapes philippinarum) is a potent antioxidant enzyme involved in antibacterial response, Fish Shellfish Immunol. 32 (2012) 513–523, https://doi.org/10.1016/J.FSI.2011.12.010.
[68] Z. Liu, H. Zhu, Y. Liu, J. Kuang, K. Zhou, F. Liang, Z. Liu, D. Wang, W. Ke, Construction of a high-density, high-quality genetic map of cultivated lotus (Nelumbo nucifera) using next-generation sequencing, BMC Genom. 17 (2016) 466, https://doi.org/10.1186/s12864-016-2781-4.
[69] C. Vorbach, R. Harrison, M.R. Capecchi, Xanthine oxidoreductase is central to the evolution and function of the innate immune system, Trends Immunol. 24 (2003) 512–517, https://doi.org/10.1016/S1471-4906(03)00237-0.
[70] G. Glantzounis, E. Tsimoyiannis, A. Kappas, D. Galaris, Uric acid and oxidative stress, Curr. Pharmaceut. Des. 11 (2005) 4145–4151, https://doi.org/10.2174/138161205774913255.
[71] Z. Khan, X.Z. Shen, E.A. Bernstein, J.F. Giani, M. Eriguchi, T.V. Zhao, R.A. Gonzalez-Villalobos, S. Fuchs, G.Y. Liu, K.E. Bernstein, X.Z. Shen, S. Fuchs, Angiotensin-converting enzyme enhances the oxidative response and bactericidal activity of neutrophils, Blood 130 (2017) 328–339, https://doi.org/10.1182/blood-2016-11-752006.
[72] S. Di Meo, T.T. Reed, P. Venditti, V.M. Victor, Role of ROS and RNS sources in physiological and pathological conditions, Oxid. Med. Cell. Longev. (2016) 1–44, https://doi.org/10.1155/2016/1245049 2016.
[73] Y. Zhou, J. Tan, Y. Dai, Y. Yu, Q. Zhang, M.E. Meyerhoff, Synthesis and nitric oxide releasing properties of novel fluoro S-nitrosothiols, Chem. Commun. 55 (2019) 401–404, https://doi.org/10.1039/C8CC08868C.
[74] F. Jeffroy, C. Paillard, Involvement of nitric oxide in the in vitro interaction between Manila clam, Ruditapes philippinarum, hemocytes and the bacterium Vibrio tapetis, Fish Shellfish Immunol. 31 (2011) 1137–1141, https://doi.org/10.1016/j.fsi.2011.10.008.
[75] C. Tafalla, B. Novoa, A. Figueras, Production of nitric oxide by mussel (Mytilus galloprovincialis) hemocytes and effect of exogenous nitric oxide on phagocytic functions, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 132 (2002) 423–431, https://doi.org/10.1016/S1096-4959(02)00050-7.
[76] P.C. Calder, Branched-chain amino acids and immunity, J. Nutr. 136 (2006) 288S–293S.
[77] P. Wongkittichote, N. Ah Mew, K.A. Chapman, Propionyl-CoA carboxylase – a review, Mol. Genet. Metabol. 122 (2017) 145–152, https://doi.org/10.1016/j.ymgme.2017.10.002.
[78] T.V. Nguyen, A.C. Alfaro, T. Young, S. Ravi, F. Merien, Metabolomics study of immune responses of New Zealand Greenshell™ mussels (Perna canaliculus) infected with pathogenic Vibrio sp, Mar. Biotechnol. 20 (2018) 396–409, https://doi.org/10.1007/s10126-018-9804-x.
[79] R.L. Taber, A. Campbell, S. Spencer, A simple experiment demonstrating the allosteric regulation of yeast pyruvate kinase, Biochem. Educ. 26 (1998) 73–76, https://doi.org/10.1016/S0307-4412(97)00117-9.
[80] A. Van Waarde, Biochemistry of non-protein nitrogenous compounds in fish including the use of amino acids for anaerobic energy production, Comp. Biochem. Physiol. Part B Biochem. 91 (1988) 207–228, https://doi.org/10.1016/0305-0491(88)90136-8.
[81] Y. Kanai, M.A. Hediger, The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects, Pflugers Arch. Eur. J. Physiol. 447 (2004) 469–479, https://doi.org/10.1007/s00424-003-1146-4.
[82] M.S. Patel, N.S. Nemeria, W. Furey, F. Jordan, The pyruvate dehydrogenase complexes: structure-based function and regulation, J. Biol. Chem. 289 (2014) 16615–16623, https://doi.org/10.1074/jbc.R114.563148.
[83] K. Brokordt, Y. Defranchi, I. Espósito, C. Cárcamo, P. Schmitt, L. Mercado, E. De la Fuente-Ortega, G.A. Rivera-Ingraham, Reproduction immunity trade-off in a mollusk: hemocyte energy metabolism underlies cellular and molecular immune responses, Front. Physiol. 10 (2019) 1–16, https://doi.org/10.3389/fphys.2019.00077.
[84] J. Flye-Sainte-Marie, An Ecophysiological Approach of Brown Ring Disease in the Manila Clam, Ruditapes Philippinarum: Experiments and Modelling, Université de Bretagne Occidentale, 2007.
[85] P. Goulletquer, A Bibliography of the Manila Clam Tapes philippinarum, IFREMER La Tremblade, France, 1997.
[86] H. Guo, G. Xu, B. Wang, F. Xia, Q. Sun, Y. Wang, E. Xie, Z. Lu, L. Jiang, Q. Xia, Phosphoenolpyruvate carboxykinase is involved in antiviral immunity against Bombyx mori nucleopolyhedrovirus, Dev. Comp. Immunol. 92 (2019) 193–198, https://doi.org/10.1016/j.dci.2018.11.015.
[87] A. Cabezas-Cruz, P.J. Espinosa, D.A. Obregón, P. Alberdi, J. de la Fuente, Ixodes scapularis tick cells control Anaplasma phagocytophilum infection by increasing the synthesis of phosphoenolpyruvate from tyrosine, Front. Cell. Infect. Microbiol. 7 (2017) 1–16, https://doi.org/10.3389/fcimb.2017.00375.
[88] E.P. Sokolov, S. Markert, T. Hinzke, C. Hirschfeld, D. Becher, S. Ponsuksili, I.M. Sokolova, Effects of hypoxia-reoxygenation stress on mitochondrial proteome and bioenergetics of the hypoxia-tolerant marine bivalve Crassostrea gigas, J. Proteomics. 194 (2019) 99–111, https://doi.org/10.1016/j.jprot.2018.12.009.
[89] C.M. Pickart, R.E. Cohen, Proteasomes and their kin: proteases in the machine age, Nat. Rev. Mol. Cell Biol. 5 (2004) 177–187, https://doi.org/10.1038/nrm1336.
[90] J. Zheng, X. Yang, J.M. Harrell, S. Ryzhikov, E.-H. Shim, K. Lykke-Andersen, N. Wei, H. Sun, R. Kobayashi, H. Zhang, CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex, Mol. Cell. 10 (2002) 1519–1526, https://doi.org/10.1016/s1097-2765(02)00784-0.
[91] G. Jubelin, T. Frédéric, D.M. Duda, Y. Hsu, A. Samba-Louaka, R. Nobe, M. Penary, C. Watrin, J.P. Nougayréde, B.A. Schulman, C.E. Stebbins, E. Oswald, Pathogenic bacteria target NEDD8-conjugated cullins to hijack host-cell signaling pathways, PLoS Pathog. 6 (2010), https://doi.org/10.1371/journal.ppat.1001128.
[92] R. Rosenzweig, P.A. Osmulski, M. Gaczynska, M.H. Glickman, The central unit within the 19S regulatory particle of the proteasome, Nat. Struct. Mol. Biol. 15 (2008) 573–580, https://doi.org/10.1038/nsmb.1427.
[93] K. Shi, J.Z. Zhang, R.L. Zhao, L. Yang, D. Guo, PSMD7 downregulation induces apoptosis and suppresses tumorigenesis of esophageal squamous cell carcinoma via the mTOR/p70S6K pathway, FEBS Open Biol. 8 (2018) 533–543, https://doi.org/10.1002/2211-5463.12394.
[94] N. González-Morales, T.K. Holenka, F. Schöck, Filamin actin-binding and titin-binding fulfill distinct functions in Z-disc cohesion, PLoS Genet. 13 (2017) 1–25, https://doi.org/10.1371/journal.pgen.1006880.
[95] M. Mrosek, D. Labeit, S. Witt, H. Heerklotz, E. von Castelmur, S. Labeit, O. Mayans, Molecular determinants for the recruitment of the ubiquitin-ligase MuRF-1 onto M-line titin, Faseb. J. 21 (2007) 1383–1392, https://doi.org/10.1096/fj.06-7644com.
[96] T. Mrowiec, B. Schwappach, 14-3-3 Proteins in membrane protein transport, Biol. Chem. 387 (2006) 1227–1236, https://doi.org/10.1515/BC.2006.152.
[97] P.E. Shaw, Peptidyl-prolyl isomerases: a new twist to transcription, EMBO Rep. 3 (2002) 521–526, https://doi.org/10.1093/embo-reports/kvf118.
[98] A. Bella, P. Monaghana, A.P. Page, Peptidyl-prolyl cis-trans isomerases (immunophilins) and their roles in parasite biochemistry, host–parasite interaction and antiparasitic drug action, Int. J. 36 (2009) 261–276.
[99] G. Van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 101 (2009) 1–4, https://doi.org/10.1038/nrm2330.Membrane.
[100] T. Imai, Y. Kato, C. Kajiwara, S. Mizukami, I. Ishige, T. Ichiyanagi, M. Hikida, J.-Y. Wang, H. Udono, Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells, Proc. Natl. Acad. Sci. Unit. States Am. 108 (2011) 16363–16368, https://doi.org/10.1073/pnas.1108372108.
[101] Y. Yik Sung, T.H. MacRae, Heat shock proteins and disease control in aquatic organisms, J. Aquacult. Res. Dev. S2 (2011), https://doi.org/10.4172/2155-9546.s2-006.
[102] N. Darbinian, G.L. Gallia, M. Kundu, N. Shcherbik, A. Tretiakova, A. Giordano, K. Khalili, Association of Purα and E2F-1 suppresses transcriptional activity of E2F-1, Oncogene 18 (1999) 6398–6402, https://doi.org/10.1038/sj.onc.1203011.
[103] B. Rocher, F. Bultelle, P. Chan, F. Foll, J. Letendre, T. Monsinjon, S. Olivier, R. Péden, A. Poret, D. Vaudry, T. Knigge, 2-DE mapping of the blue mussel gill proteome: the usual suspects revisited, Proteomes 3 (2015) 3–41, https://doi.org/10.3390/proteomes3010003.
[104] D.H. Kang, K.Y. Song, H.S. Choi, P.Y. Law, L.N. Wei, H.H. Loh, Novel dual-binding function of a poly (C)-binding protein 3, transcriptional factor which binds the double-strand and single-stranded DNA sequence, Gene 501 (2012) 33–38, https://doi.org/10.1016/j.gene.2012.04.001.
[105] R. Wongpanya, T. Aoki, I. Hirono, M. Yasuik, A. Tassanakajon, Analysis of gene expression in haemocytes of shrimp Penaeus monodon challenged with white Spot syndrome virus by cDNA microarray, Sci. Asia 33 (2007) 165–174, https://doi.org/10.2306/scienceasia1513-1874.2007.33.165.
[106] E. David, A. Tanguy, K. Pichavant, D. Moraga, Response of the Pacific oyster Crassostrea gigas to hypoxia exposure under experimental conditions, FEBS J. 272 (2005) 5635–5652, https://doi.org/10.1111/j.1742-4658.2005.04960.x.