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Transcriptome-wide analysis of wild Asari (=Manila) clams affected by the Brown Muscle Disease: Etiology and impacts of the disease

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ABSTRACT

Recently, we reported an emerging pathology named Brown Muscle Disease (BMD) affecting Asari clams inhabiting the most productive area for this species in France, the Arcachon Bay. The main macroscopic feature of the pathology relies on the atrophy of the posterior adductor muscle, affecting the ability of clams to burry. The research of the etiological agent of BMD privileged a viral infection. Contrary to healthy clams, infected animals are always found at the surface of the sediment and exhibit 30 nm virus-like particles in muscle, granulocytic and rectal cells. In order to get more insights on the etiology and impacts of the BMD on clams, we took advantage in the present study of next generation sequencing technologies. An RNA-Seq approach was used (i) to test whether viral RNA sequences can be specifically found in the transcriptome of diseased animals and (ii) to identify the genes that are differentially regulated between diseased and healthy clams. Contrary to healthy buried animals, in diseased clams one sequence showing extensive homologies with retroviridae-related genes was detected. Among the biological processes that were affected in diseased clams, the synaptic transmission process was the most represented. To deepen this result, a new sampling was carried out and the transcription level of genes involved in synaptic transmission was determined in healthy and diseased clams but also in clams with no visible sign of pathology but located at the surface of the sediment. Our findings suggest that muscle atrophy is a latter sign of the pathology and that nervous system could be instead a primary target of the BMD agent.

1. Introduction

Asari (=Manila) clam (*Ruditapes philippinarum*) is a worldwide exploited bivalve native from Indo-Pacific region [1–3]. The loss of production is preoccupying in countries where Asari clams were introduced like in Canada (50% drop between 2012 and 2014) or Italy (5% decrease between 2007 and 2014 (FAO 2014)). There is a common agreement to point out that in most cases infectious diseases are a major cause of mortality [4]. This is consistent with the general idea that infectious diseases represent the first cause of mortality in aquaculture [5], although in the case of Asari clam sources of production can be aquaculture and/or fishing.

The most commonly recorded pathogens of Asari clam are the prokaryotic *Vibrio tapetis* [6] and the alveolate *Perkinsus olseni* [7,8], although some metazoans can locally impact clam populations like the pycnogonid sea spider *Nymphonella tapetis* [9,10] and trematode species [11,12]. Few years ago, a pathology named Brown Muscle Disease (BMD) was described in Arcachon Bay [13], the most productive French area for Asari clam (50% of capture) [2]. BMD symptoms were exhaustively described, the main macroscopic feature being the necropsies of the posterior adductor muscle [13,14]. A questioning point was the lack of impact on the anterior adductor muscle which presents roughly the same histologic organization [15]. Besides, the propagation of the necrosis does not seem to be random, developing from the striated muscle part of the muscle to rapidly invade the totality of the muscle (thus including smooth muscle part) [13]. BMD had also heavy consequences on the general fitness of the clam, with significant negative effect on the Asari clam index condition (flesh weight versus shell weight ratio) [14–16]. The research of the etiological agent of BMD suggested a viral infection, based on histology and transmission electron microscopy observations [17]. All infected clams exhibited electron-dense particles of 25–35 nm in all tissues, in contrast with healthy clams in which no virus-like particles were detected. These virus-like particles were isolated, but however we were unable to experimentally infect clams (unpublished data). Clinical and histological observations were different from the few previous studies describing viral or viral-like infections in adults of *Ruditapes* spp. Clams [18–21].

In the present study, in order to get more insights on the etiology

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and impacts of the BMD on clams, we used a large scale without a priori RNA-Seq based approach. More specifically, RNA-Seq approach was used to (i) test whether viral RNA sequences can be specifically found in the transcriptome of diseased clams [22] and (ii) identify the genes, and by extension, biological functions that are differentially regulated between diseased and healthy clams. The muscle transcriptome of specimens was determined by high throughput RNA sequencing using Illumina HiSeq 2000 technology. From these results, a new sampling was carried out to collect diseased and healthy animals but also animals with no visible sign of BMD and located at the surface of the sediment. The transcription level of a reduced number of genes previously highlighted by the RNA-Seq study and involved in synaptic transmission was determined by quantitative RT-PCR.

2. Material and methods

2.1. Wild clam sample collection

For RNA-Seq analyses, specimens of *R. philippinarum* were collected from an intertidal site (44°41′N, 01°04′W) of the Arcachon Bay, which characteristics were described in Ref. [16]. Manila clams were sampled on a restricted area (a few dozen meters few meters) in autumn 2012. Two following status were sampled: buried (BUR) animals with no sign (−) of Brown Muscle Disease BMD ($BUR^{(-)}$) and clams at the surface (SURF) of the sediment exhibiting signs (+) of BMD ($SURF^{(+)}$). Each sampled clam was opened with a scalpel and BMD occurrence was estimated by eye. BMD intensity was assessed through the Muscle Print Index (MPI). MPI characterizes the percentage of the posterior adductor muscle surface colonized by the brown muscle on a scale of 0–4 as follows: 0 (healthy = without BMD), 1 (0–25% of the muscle surface is affected), 2 (25–50%), 3 (50–75%) and 4 (75–100%) [13]. Two $BUR^{(-)}$ clams (MPI = 0, length = 30 mm) and three $SURF^{(+)}$ clams (MPI = 3, length = 32 mm) were immediately dissected. Posterior adductor muscle were immediately fixed in RNAlater solution and stored at −80 °C until needed for analyses.

A second sampling was carried out in spring 2016. In addition to $BUR^{(-)}$ and $SURF^{(+)}$ animals, animals with no visible sign of BMD but located at the surface of the sediment ($SURF^{(-)}$) were also collected. Ten animals of each group were sampled, observed and dissected as previously described. Posterior adductor muscle were immediately fixed in RNAlater solution and stored at −80 °C until needed for quantitative PCR analyses.

2.2. Preparation of cDNA libraries, contig assembly and RNA-Seq data analyses

Samples of posterior adductor muscle were homogenized by means of a bead mill homogenizer using ceramic beads (40 s, MP Biomedicals) in 500 µL of Trizol reagent. Total RNAs were extracted using the RNeasy Mini kit (Qiagen). During this step, samples were submitted to DNAseI treatment, according to the manufacturer's instructions. A total of 5 clams were used, i.e. 1 pool of 3 $SURF^{(+)}$ clams and 1 pool of 2 $BUR^{(-)}$clams. Then, preparation of cDNA libraries for Illumina HiSeq 2000 sequencing was done using the Truseq RNA sample preparation v2 kit (Illumina), following the manufacturer's instructions. The two individually tagged libraries (one library per pool, $BUR^{(-)}$ and $SURF^{(+)}$) were pooled in equal amounts and sequenced on 1 lane at the Genome and Transcriptome Platform of Toulouse (Genotoul, France) using Illumina HiSeq 2000 technology (100 bp paired-ends reads). The raw reads have been deposited in the NCBI's Gene Expression Omnibus database [23] under accession number GSE119518.

Base-calling was performed using the ng6 processing environment [24]. Sequence quality was checked using the Burrows-Wheeler Aligner and fastQC software [25]. *De novo* assembling was carried out using the Oases software [26] and the Velvet algorithm [27]. Chimeric sequences and sequences with a length inferior to 200 bp were discarded. Transcriptome quality was assessed with BUSCO v3 [28]. BUSCO provides a quantitative assessment of a transcriptome assembly quality by looking at the completeness in terms of its expected gene content. Here, our assembly was assessed against the Metazoa orthologs dataset. The quality of our assembly was evidenced by the high abundance (94.2%) of complete Metazoa benchmarking universal single-copy orthologues (BUSCO summary: C:94.2%[S:81.7%,D:12.5%],F:0.3%,M:5.5%,n:978). To annotate the contigs based on similarity with known proteins, contigs were blasted on the nr protein database using BLAST program. Gene transcription level was normalized by using RPKM (Reads Per Kilobase per Million mapped reads; [29]) before functional analyses.

Functional classification and assessment of significant differential representation of functional classes were performed with the Blast2go software [30] using Gene Ontology annotation and the Fisher's exact test (enrichment analysis). To do this a reference list of genes was constructed. Repetitive contigs (i.e. multiple contigs that had the same annotation), non-annotated contigs or contigs with low homology (Evalue $> 10^{-10}$) were discarded. A total of 8414 unique genes of known function were identified and were used as a reference. From this reference list, the test lists were constituted by the genes that were down-regulated (fold change < 0.2) and/or up-regulated (fold change > 5) in diseased ($SURF^{(+)}$) compared to healthy ($BUR^{(-)}$) clams.

2.3. Quantitative RT-PCR analyses

A muscle sample of 30 mg (wet weight) was homogenized in 600 µL of ice-cold RTL buffer (Qiagen) with 6 µL of β-mercaptoethanol using a tissue homogenizer for 30s (Mixer Mill MM 200, Retsch). Following centrifugation, RNA was extracted from the homogenate using the AllPrep DNA/RNA kit (Qiagen) according to manufactures' guidelines. In order to avoid a potential contamination of RNA by DNA, total RNA was treated with DNAseI (Qiagen) according to the manufacturer's recommendations.

For each sample, RNA quality was evaluated by electrophoresis on a 1% agarose gel and concentrations as well as purity were determined by spectrophotometry (Take 3, Epoch, Biotek). First-strand cDNA was synthesized from total RNA using the GoScript Reverse Transcription System (Promega), according to the manufacturer's instructions. Following the reverse transcriptase reaction, cDNA was diluted 10-fold. Real-time PCR reactions were then performed in an MX3000P (Stratagene; 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and 72 °C for 30 s). Each 20 µL reaction contained 12.5 µL of GoTaq qPCR master mix (Promega), 5 µL template and the specific primer pairs at a final concentration of 250 nM each. Specific primer pairs were designed by means of the Primer3plus software (Table S1). The reaction specificity was determined for each reaction by gel electrophoresis and from the dissociation curve of the PCR product. This was obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. Amplification efficiencies for all primer sets were calculated; all values proved to be sufficient to allow direct comparison of amplification plots according to the ΔΔCt method [31]. Relative quantification of gene expression was achieved by concurrent amplification of the *cilia- and flagella-associated protein 20* gene (*cfap20*) and the *cleavage and polyadenylation specificity factor subunit 5* gene (*nutd21*). Indeed, the transcription of these two genes was found to be unaffected by BMD in the previous RNA-Seq analysis (i.e. their fold change between $SURF^{(+)}$ and $BUR^{(-)}$ clams was equal to 1). The gene *nutd21* was found to be the most relevant under our conditions and was finally used as endogenous control.

2.4. Statistical analyses

Comparisons among clams groups were performed by analysis of variance (ANOVA), after checking assumptions of normality (Kolmogorov-Smirnov) and homoscedasticity of the error terms

(Levene). When the assumptions were not met as deduced from ad-hoc tests, we used box-cox data transformations or the nonparametric Kruskal – Wallis test. If significant effects were detected, the Least Square Deviation (LSD) or U-Mann – Whitney tests were used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using STATISTICA version 6.1 software (StatSoft) and XLSTAT (Addinsoft version 2012.6.08). Numerical results are given as means ± SE.

3. Results

3.1. RNA-seq data

RNA-seq generated 342 million fragments averaging 75 bases in length. The assembly of these reads generated a total of 47,339 contigs, with a mean size of 2392 bp (N50 = 3105). A total of 15,849 contigs showed homology with known sequences (BLASTX, Evalue $\leq 10^{-10}$). Finally, a total of 8414 unique genes of known function were identified (i.e. multiple contigs that had the same annotation were removed and only the hit with the best Evalue was retained for each gene).

3.2. Viral sequences

Among all the determined contigs only 285 were recovered in $SURF^{(+)}$ individuals. Compared to databases using the Blast algorithm, forty of these contigs evidenced homologies with known proteins. One of them, RPHIL_POL2.4.16, showed extensive homologies with genes that are classically encountered in retroviridae sequences (Fig. 1). Indeed, these fragment of 2637 bp (accession number MG570405) encoded successively for complete reverse transcriptase (RT-LTR), RNase-H and retroviral integrase (RVE) proteins.

3.3. Impacts of the BMD on clam's transcriptome

Analyses were carried out to identify the genes that were differentially regulated (by at least a factor 5) between diseased ($SURF^{(+)}$) and healthy animals ($BUR^{(-)}$). A total of 358 unique genes were identified, with 206 genes being up-regulated (Table S2) and 152 genes being down-regulated (Table S3) in $SURF^{(+)}$ compared to $BUR^{(-)}$ clams. An enrichment analysis with Fisher's exact test ($p < 0.01$) was performed on these differentially expressed genes to highlight the most significant biological processes that differed between the two groups of animals (Fig. 2). The principal functions represented among the 358 differentially transcribed genes are summarized in Fig. 2A. Concerning the biological processes and molecular functions associated to the down-regulated genes in diseased animals (Fig. 2B), the synaptic transmission process (GO terms: regulation of excitatory postsynaptic membrane potential, negative regulation of synaptic transmission, learning, cholinesterase activity, terminal bouton and in a lesser extent cellular calcium homoeostasis) was the most represented. It is noteworthy that this process was the only one that was highlighted at a more stringent threshold (FDR < 0.05, see Fig. 3). No significant result was obtained at this threshold with the list of up-regulated genes (Fig. 2C). The other down-regulated functions were related to the inflammatory response (GO terms: cell migration, response to glucocorticoid stimulus), to blood coagulation (GO terms: positive regulation of blood coagulation, fibrinolysis) and to cell differentiation and division (GO terms: positive regulation of ERK1 and ERK2 cascade, tyrosine metabolic process, skeletal muscle fiber development). Biological processes and molecular functions associated to the up-regulated genes in diseased animals were related to the immune response (GO terms: embryonic hemopoiesis, retinoic acid metabolic process), oxidative stress (GO term: glutathione peroxidase activity), development and morphogenesis (GO terms: embryonic forelimb morphogenesis, retinoic acid metabolic process) and cellular amide metabolic process.

3.4. Impacts of the BMD on genes involved in synaptic transmission

In addition to $BUR^{(-)}$ and $SURF^{(+)}$ clams, animals with no visible sign of BMD but located at the surface of the sediment ($SURF^{(-)}$) were collected in spring 2016. We determined the transcription level of three genes involved in synaptic transmission, i.e. *bche* (cholinesterase precursor), *grik2* (glutamate receptor kainate 2-like) and *ppp3ca* (serine threonine-protein phosphatase 2b catalytic subunit alpha isoform isoform 2) by quantitative RT-PCR in the posterior adductor muscle of individuals. The transcription level of *bche*, *grik2* and *ppp3ca* was found to be significantly down-regulated in $SURF^{(+)}$ clams in comparison to $BUR^{(-)}$ clams (Fig. 4). In addition, $SURF^{(-)}$ clams presented intermediate values between $BUR^{(-)}$ and $SURF^{(+)}$ clams. The transcription level of *bche* and *ppp3ca* was significantly lower in $SURF^{(-)}$ clams in comparison to $BUR^{(-)}$ clams. In addition, for the three genes analyzed, no significant difference was observed between $SURF^{(-)}$ and $SURF^{(+)}$ clams.

4. Discussion

Numerous viruses belonging to different families (*Herpesviridae, Papovaviridae, Togaviridae* for example) have been previously reported as infecting marine molluscs [22,32]. Most of them were identified in cultivated organisms for aquaculture like oysters, mussels, scallops or abalones. However, there is still a lack of molecular information concerning these viruses, mainly due to their identification which is classically based on histological studies. Indeed, few marine viral genomes have been completely characterized and most of the time only those having important economic impact have been extensively studied. New generation sequencing, using high throughput approaches like RNA-Seq, has started revealing the huge diversity of marine viruses and has been evidenced to be a useful tool to identify and characterize virus sequences in their host [22,33,34]. In the present study, among all the contigs assembled from the derived-RNA-Seq sequences, we identified one contig presenting extensive homologies with retroviruses genes *rt-ltr, rnase-H* and *rve*. Theses retrovirus-related sequences were present only in infected clams. Retroviruses genomes encode structural and enzymatic viral proteins. They are usually organized as: 5'LTR-*gag-pol-env*-3'LTR [35,36]. In these genomes *gag* encodes for the nucleocapsid protein and *env* for the viral envelope glycoprotein, while the *pol* gene encodes for a reverse transcriptase, a RNase-H and an integrase proteins. These extensive homologies with the POL polyproteins and the fact that this fragment has been only encountered in diseased clams suggest that the RPHIL_POL2.4.16 fragment could represent a part of the genome from the etiologic agent of the BMD. This finding is consistent with previous description of the BMD agent where virus like particles (VLPs) of around 30 nm have been evidenced [17]. However, future prospects using molecular approaches will be necessary to unequivocally relate this virus to a family.

Concerning the impacts of the BMD on the muscle transcriptome of

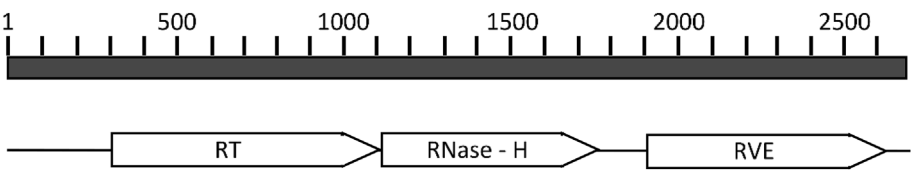

Fig. 1. Proteins encoded by the RPHIL_POL2.4.16 contig showing homologies with retroviruses pol gene products. RT = Reverse transcriptase; RVE = Retrovirus integrase.

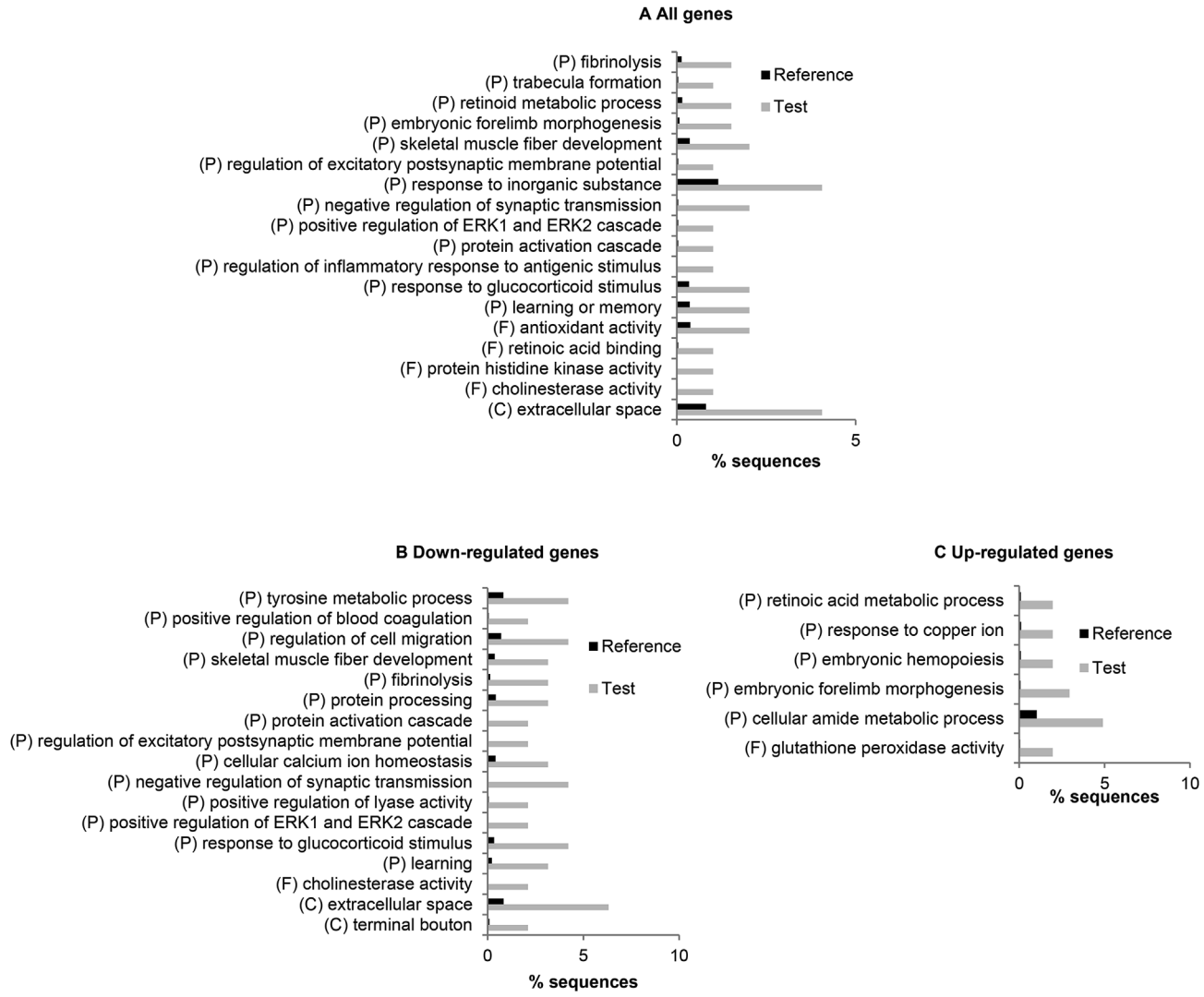

Fig. 2. Significantly enriched biological processes (P), molecular function (F) and cellular component (C) reduced to the most specific terms in diseased (SURF(+)) versus healthy animals (BUR(−); Fisher's exact test, significance threshold: $P < 0.01$). Diagram 1A was built with the 359 genes differentially expressed by at least a factor 5 between conditions, diagram 1B describes the down-regulated genes (fold change < 0.2) in diseased animals and diagram 1C describes the up-regulated genes (fold change > 5).

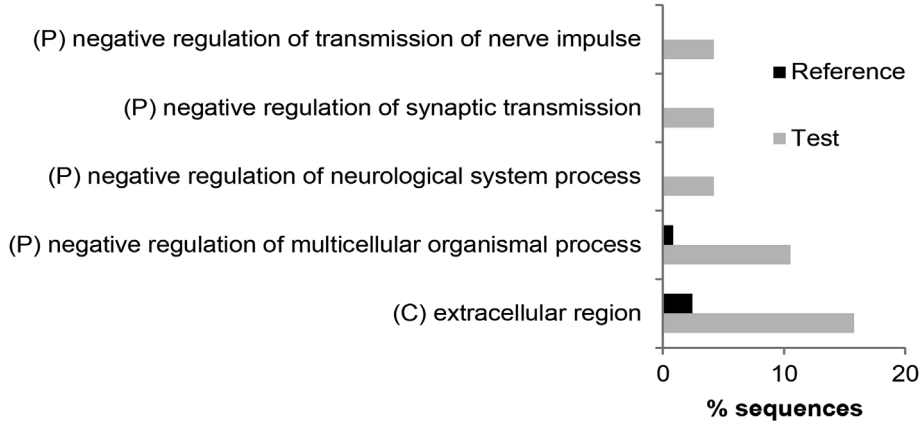

Fig. 3. Significantly enriched biological processes (P) and cellular component (C) reduced to the most specific terms in diseased (SURF(+)) versus healthy animals (BUR(−); Fisher's exact test, significance threshold: FDR < 0.05). The Diagram was built with the 152 down-regulated genes by at least a factor 5 between conditions.

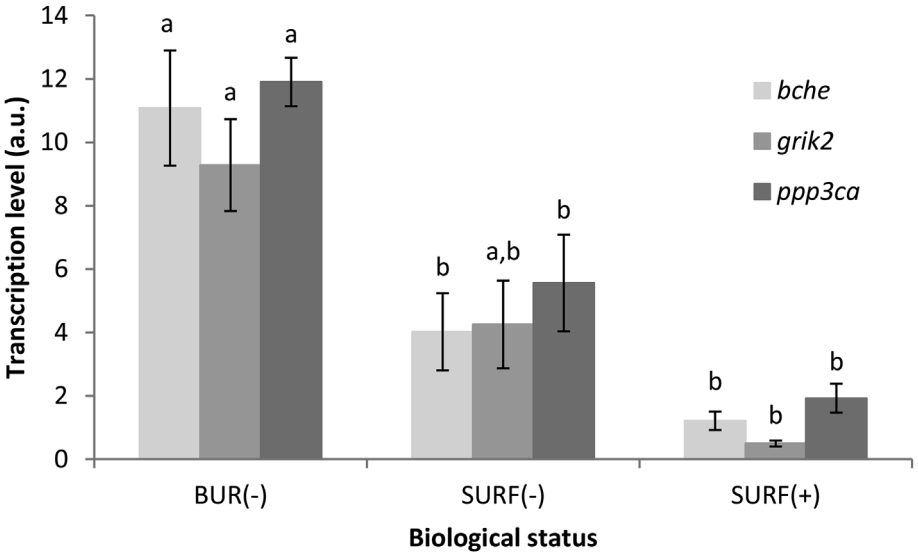

Fig. 4. Change in the transcription levels (mean ± SE; n = 10) of *bche*, *grik2* and *ppp3ca* in the posterior adductor muscle of clams collected in spring 2016 and presenting different biological status (BUR(−), SURF(−), SURF(+)). Bars sharing same-case letters do not differ significantly ($P > 0.05$).

clams, according to our previous findings at both the transcriptional and histological levels [13,16,17], we found an up-regulation of genes involved in the defense against oxidative stress (GO term: glutathione peroxidase activity with the genes: glutathione peroxidase 1, fold change (FC) 11.1; chorion peroxidase, FC 11.5; glutathione peroxidase 2, FC 13.1). In the same way, we observed significant changes in the transcription levels of inflammatory- and immune-related genes. Genes involved in the immune or inflammatory response (Fig. 1) were both up- and down-regulated between $SURF^{(+)}$ and $BUR^{(-)}$ clams. For example, among the most down-regulated genes, we found two genes encoding for proteins that are two major actors of the innate immunity, the component complement C3 (FC 0.07) and the big defensing 3 (FC 0.1) [37,38]. Among the most up-regulated genes, we found a gene involved in the inflammation (the gene tyrosine-protein kinase Lck, FC 12.1; [39]) and two other genes known to be involved in the host defense during pathogen infection (the gene pathogenesis-related thaumatin-like protein 1, FC 11.5 and the gene tandem repeat galectin, FC 11.8; [40,41]. Similar findings were also reported in diverse bivalve species infected by bacteria or protozoans (see Ref. [40]). For authors, whereas some transcripts involved in the immune or inflammatory response are up-regulated, others are in the same time switched down, providing a tailored response to the pathogen [40]. An alternative hypothesis in our case could be that the BMD agent deregulates the clam defense. Indeed, the persistence of a virus in a host depends on its ability to evade and/or deregulate the host defense. Numerous viral mechanisms causing immune evasion were described in order to establish virus persistence, including a down-regulation of complement C3 mRNA transcription [42,43]. We also found that several genes involved in skeletal muscle fiber development were down-regulated. This is in accordance with that fact that the BMD triggers posterior adductor muscle atrophy [13].

Most surprisingly, among the biological processes that were affected in diseased animals in comparison to healthy animals, the synaptic transmission process was the most represented. Genes involved in synaptic transmission (e.g. cholinesterase precursor (*bche*), cholinesterase isoform ×2, glutamate receptor kainate 2-like (*grik2*), serine threonine-protein phosphatase 2b catalytic subunit alpha isoform isoform 2 (*ppp3ca*), serum response factor, ankyrin isoform u) were down-regulated. Moreover, the down-regulated gene FRMFamide (FC 0.1) encodes for a neuropeptide that have been proven to be especially useful to visualize the developing nervous system in molluscs [44]. We must note however that one gene involved in synaptic transmission was found to be up-regulated (FC 6.4), the gene encoding for the 5-hydroxytryptamine receptor 2a. Moreover, among the up-regulated functions identified, several could be associated to nervous system development. For example, in the class retinoic acid metabolic process, two up-regulated genes, i.e. cellular retinoic acid-binding protein 1 and 2 (FC 10.1 and 12.5, respectively), were found to be involved in the development and differentiation of the mammalian nervous system [45]. Moreover, in the class embryonic forelimb morphogenesis, the up-regulated gene homeobox protein engrailed-1 (FC 6) was found to be involved in the development of the central nervous system in mammals [46]. In bivalves, the nervous system is made up by three pairs of ganglia, the cerebropleural ganglia, the pedal ganglia and the visceral ganglia. The visceral ganglia are located on the surface of the posterior adductor muscle. Visceral ganglia not only control the posterior adductor muscle but also the posterior foot retractor muscles, gills, heart pericardium, kidney, the posterior region of the digestive tract, gonad, the mantle, siphons and pallial sense organs. In contrast to the posterior muscle, the anterior adductor muscle does not present ganglia on its surface but is innervated by nervous projections from the cerebropleural ganglia [47]. It is noteworthy that the BMD only affects the posterior adductor muscle of animals, leading to its atrophy. The anterior muscle was never found to be affected in either diseased or healthy animals [13,17]. Transcriptomic data coupled with previous anatomopathological findings could suggest that the nervous system and notably visceral ganglia are a main cellular target of the BMD pathogen. Among the deregulated genes previously evoked, several are known to be involved in the control of muscle contraction or relaxation. The up-regulated gene *htr2a* encodes for a receptor for serotonin. Serotonin is involved in muscle relaxation and valve opening in bivalves [48]. Three genes encoding for proteins involved in the metabolism/pathway of excitatory neurotransmitters glutamate and acetylcholine (i.e. *bche*, *grik2* and in a lesser extent *ppp3ca*) were down-regulated in diseased animals [48,49]. Cholinesterase is the enzyme responsible for the inactivation of acetylcholine, a neurotransmitter known to trigger muscle adductor contraction and subsequently valves closure in bivalves [48]. Valve closure is indeed an active mechanism while opening is achieved passively by ligaments. Valve closure is required to protect animals from predators or contaminants [50], to expulse pseudofaeces, but also in clams, in association with the foot and siphons, to bury [51,52]. We previously reported a reducing ability of BMD clams to bury [13]. In the case of BMD animals, this could be linked to the atrophy of posterior adductor muscle. However, although *R.*

phillippinarum typically lives buried in the sediment, BMD clams are always found at the surface of the sediment in association with other Asari clams with no visible sign of BMD ($SURF^{(-)}$ clams). In our previous works, we found that $SURF^{(-)}$ animals presented intermediate transcription level of genes involved in immune response, in mitochondrial metabolism or the oxidative stress response as well as intermediate phagocytosis capacity and intermediate condition index between $BUR^{(-)}$ and $SURF^{(+)}$ clams, suggesting that muscle atrophy (i.e. BMD) could be a latter sign of the pathology [16]. Taken together, these results could suggest that the nervous system and more specifically neuromuscular junctions could be a primary site of action of the BMD agent. In support of this hypothesis, in a study carried out on the freshwater bivalve *Corbicula Fluminea*, Cooper and Bidwell [53] have shown that cholinesterase inhibition reduces the capacity of animals to burrow into the substrate. Moreover, muscle atrophy and calcification, anatomopathological characteristics of the BMD [17], are known post-syndromes of neuromuscular disorders [54].

In order to gain a deeper insight into such hypothesis, we came back in the field (year 2016) to collect $BUR^{(-)}$, $SURF^{(-)}$ and $SURF^{(+)}$ animals. We then determined the gene transcription level of *bche*, *grik2* and *ppp3ca* by quantitative RT-PCR in the posterior adductor muscle of individuals. According to previous results obtained by RNA-Seq in $BUR^{(-)}$ and $SURF^{(+)}$ individuals collected in year 2012, the transcription level of *bche*, *grik2* and *ppp3ca* was found to be significantly down-regulated in $SURF^{(+)}$ clams in comparison to $BUR^{(-)}$ clams. In addition, $SURF^{(-)}$ clams presented intermediate values between $BUR^{(-)}$ and $SURF^{(+)}$ animals. These findings reinforce the hypothesis that muscle atrophy (i.e. BMD) is a latter sign of the pathology and that nervous system could be instead a primary target of the BMD agent. Interestingly, we previously reported the presence of free unenveloped virus-like particles (VLPs) in the cytoplasm of rectal cells of BMD clams, suggesting direct penetration. It is intriguing to speculate that the infectious agent penetrates *via* the digestive system before affecting motor neurons, leading *in fine*, and perhaps only in some cases, to muscle atrophy. However, cautions must be taken. We cannot exclude that other environmental factors (e.g. organic contaminants or metals such as manganese, chromium or nickel) or pathogens or even a combination of them could be responsible for the dysregulation of genes involved in the nervous or immune system [55]. In this view, the BMD agent could be an opportunistic pathogen, affecting already moribund individuals unable to bury (i.e. SURF(−) clams).

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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.2018.11.043.

References

[1] S. Chiesa, L. Lucentini, R. Freitas, F. Nonnis Marzano, S. Breda, E. Figueira, N. Caill-Milly, R.J.H. Herbert, A.M.V.M. Soares, E. Argese, A history of invasion: *COI* phylogeny of Manila clam *Ruditapes philippinarum* in Europe, Fish. Res. 186 (2017) 25–35.

[2] X. de Montaudouin, I. Arzul, N. Caill-Milly, A. Khayati, J.-M. Labrousse, C. Lafitte, C. Paillard, P. Soudant, P. Goulletquer, Asari clam (*Ruditapes philippinarum*) in France: history of an exotic species 1972-2015, Bull. FRA 42 (2016) 35–42.

[3] J.-P. Flassch, Y. Leborgne, Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement, ICES Mar. Sci. Symp. 194 (1992) 92–96.

[4] S. Watanabe, J. Higano, Outline of the third international symposium on Manila (asari) clam, Bull. FRA 42 (2016) 7–8.

[5] R.B. Carnegie, Effects in mollusc culture, in: K. Rohde (Ed.), Marine Parasitology, CSIRO & CABI, Collingwood, Australia, 2005, pp. 391–398.

[6] C. Paillard, S. Gausson, J.-L. Nicolas, J.-P. le Pennec, D. Haras, Molecular identification of *Vibrio tapetis*, the causative agent of the brown ring disease of *Ruditapes philippinarum*, Aquaculture 253 (2006) 25–38.

[7] F. Ruano, F.M. Batista, Perkinsosis in the clams *Ruditapes decussatus* and *R. philippinarum* in the Northeastern Atlantic and Mediterranean sea: a review, J. Invertebr. Pathol. 131 (2015) 58–67.

[8] P. Soudant, F.-L. Chu, A. Volety, Host-parasite interactions: marine bivalve molluscs and protozoan parasites, *Perkinsus* species, J. Invertebr. Pathol. 114 (2013) 196–216.

[9] M. Toba, S. Kobayashi, J. Kakino, H. Yamakawa, R. Ishii, R. Okamoto, Stocks and fisheries of asari in Japan, Bull. FRA 42 (2016) 9–21.

[10] T. Tomiyama, K. Yamada, K. Wakui, M. Tamaoki, K. Miyazaki, Impact of sea spider parasitism on host clams: relationships between burial patterns and parasite loads, somatic condition and survival of host, Hydrobiologia 770 (2016) 15–26.

[11] C. Dang, X. de Montaudouin, J. Bald, F. Jude, N. Raymond, L. Lanceleur, I. Paul-Pont, N. Caill-Milly, Testing the enemy release hypothesis: trematode parasites in the non indigenous Manila clam *Ruditapes philippinarum*, Hydrobiologia 630 (2009) 139–148.

[12] T. Endo, T. Hoshina, Redescription and identification of a Gymnophallid Trematode in a brackish water clam, *Tapes (Ruditapes) philippinarum*, Jpn. J. Parasitol. 23 (1974) 73–77.

[13] C. Dang, X. de Montaudouin, P. Gonzalez, N. Mesmer-Dudons, N. Caill-Milly, Brown muscle disease (BMD), an emergent pathology affecting Manila clam *Ruditapes philippinarum* in Arcachon Bay (SW France), Dis. Aquat. Org. 80 (3) (2008) 219–228.

[14] C. Dang, X. de Montaudouin, Brown muscle disease and Manila clam *Ruditapes philippinarum* dynamics in Arcachon Bay, France, J. Shellfish Res. 28 (2009) 355–362.

[15] C. Dang, X. de Montaudouin, N. Savoye, N. Caill-Milly, P. Martinez, P.-G. Sauriau, Stable isotopes changes in the adductor muscle of diseased marine bivalve *Ruditapes philippinarum*, Mar. Biol. 156 (2009) 611–618.

[16] C. Binias, P. Gonzalez, M. Provost, C. Lambert, X. de Montaudouin, Brown muscle disease: impact on Manila clam *Venerupis* (= *Ruditapes*) *philippinarum* biology, Fish Shellfish Immunol. 36 (2) (2014) 510–518.

[17] C. Dang, P. Gonzalez, N. Mesmer-Dudons, J.R. Bonami, N. Caill-Milly, X. de Montaudouin, Virus-like particles associated with brown muscle disease in Manila clam, *Ruditapes philippinarum*, in Arcachon Bay (France), J. Fish. Dis. 32 (7) (2009) 577–584.

[18] G. Arcangeli, C. Terregino, P. De Benedictis, B. Zecchin, A. Manfrin, E. Rossetti, C. Magnabosco, M. Mancin, A. Brutti, Effect of high hydrostatic pressure on murine norovirus in Manila clams, Lett. Appl. Microbiol. 54 (2012) 325–329.

[19] K.S. Bateman, P. White, M. Longshaw, Virus-like particles associated with mortalities of the Manila clam *Ruditapes philippinarum* in England, Dis. Aquat. Org. 99 (2012) 163–167.

[20] B. Novoa, A. Figueras, Virus-like particles associated with mortalities of the carpetshell clam *Ruditapes decussatus*, Dis. Aquat. Org. 39 (2000) 147–149.

[21] E. Volpe, N. Pagnini, P. Serratore, S. Ciulli, Fate of redspotted grouper nervous necrosis virus (RGNNV) in experimentally challenged Manila clam Ruditapes philippinarum, Dis. Aquat. Org. 125 (2017) 53–61.

[22] I. Arzul, S. Corbeil, B. Morga, T. Renault, Viruses infecting marine molluscs, J. Invertebr. Pathol. 147 (2017) 118–135.

[23] R. Edgar, M. Domrachev, A.E. Lash, Gene Expression Omnibus: NCBI gene expression and hybridization array data repository, Nucleic Acids Res. 3 (2002) 207–210.

[24] J. Mariette, F. Escudié, N. Allias, G. Salin, C. Noirot, S. Thomas, et al., NG6: integrated next generation sequencing storage and processing environment, BMC Genomics 13 (2012) 462.

[25] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform, Bioinformatics 25 (14) (2009) 1754–1760.

[26] M.H. Schulz, D.R. Zerbino, M. Vingron, E. Birney, Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels, Bioinformatics 28 (8) (2012) 1086–1092.

[27] D.R. Zerbino, E. Birney, Velvet: algorithms for de novo short read assembly using de Bruijn graph, Genome Res. 18 (2008) 821–829.

[28] F.A. Simão, R.M. Waterhouse, P. Ioannidis, E.V. Kriventseva, E.M. Zdobnov, BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs, Bioinformatics 31 (2015) 3210–3212.

[29] A. Mortazavi, B. Williams, K. McCue, Mapping and quantifying mammalian transcriptomes by RNA-Seq, Nat. Methods 5 (7) (2008) 621–628.

[30] A. Conesa, S. Götz, J.M. García-Gómez, J. Terol, M. Talón, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (18) (2005) 3674–3676.

[31] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2ΔCT Method, Methods 25 (4) (2001) 402–408.

[32] T.R. Meyers, T. Burton, W. Evans, N. Starkey, Detection of viruses and virus-like particles in four species of wild and farmed bivalve molluscs in Alaska, USA, from 1987 to 2009, Dis. Aquat. Org. 88 (2009) 1–12.

[33] J.R. Brum, J.C. Ignacio-Espinoza, S. Roux, G. Doulcier, S.G. Acinas, A. Alberti, S. Chaffron, C. Cruaud, C. de Vargas, J.M. Gasol, G. Gorsky, A.C. Gregory, L. Guidi, P. Hingamp, D. Iudicone, F. Not, H. Ogata, S. Pesant, B.T. Poulos, S.M. Schwenck, S. Speich, C. Dimier, S. Kandels-Lewis, M. Picheral, S. Searson, Tara Oceans Coordinators, P. Bork, C. Bowler, S. Sunagawa, P. Wincker, E. Karsenti,

M.B. Sullivan, Ocean plankton Patterns and ecological drivers of ocean viral communities, Science 348 (6237) (2015).
[34] J. Martínez Martínez, B.K. Swan, W.H. Wilson, Marine viruses, a genetic reservoir revealed by targeted viromics, ISME J. 8 (5) (2014) 1079–1088.
[35] L. Balvay, M. Lopez Lastra, B. Sargueil, J.L. Darlix, T. Ohlmann, Translational control of retroviruses, Nat. Rev. Microbiol. 5 (2007) 128–140.
[36] J. Leblanc, J. Weil, K. Beemon, Posttranscriptional regulation of retroviral gene expression: primary RNA transcripts play three roles as pre-mRNA, mRNA, and genomic RNA, Interdiscip. Rev. RNA 4 (2013) 567–580.
[37] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[38] L. Song, L. Wang, L. Qiu, H. Zhang, Bivalve immunity, in: Kenneth Söderhäll (Ed.), Invertebrate Immunity, Landes Bioscience and Springer Science, 2010, pp. 44–65.
[39] M. Gaeste, A. Kotlyarov, M. Kracht, Targeting innate immunity protein kinase signalling in inflammation, Nat. Rev. Drug Discov. 8 (6) (2009) 480–499.
[40] 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 (1) (2014) 2–11.
[41] B. Petre, I. Major, N. Rouhier, S. Duplessis, Genome-wide analysis of eukaryote thaumatinlike proteins (TLPs) with an emphasis on poplar, BMC Plant Biol. 15 (2011) 11–33.
[42] K. Stoermer, T.E. Morrison, Complement and viral pathogenesis, Virology 411 (2011) 362–373.
[43] K. Meyer, S.K. Bose, A.M. Di Bisceglie, R.B. Ray, R. Ray, Hepatitis C virus proteins inhibit C3 complement production, J. Virol. 86 (4) (2012) 2221–2228.
[44] V. Dyachuk, N. Odintsova, Development of the larval muscle system in the mussel *Mytilus trossulus* (Mollusca, Bivalvia), Dev. Growth Differ. 51 (2009) 69–79.
[45] M. Maden, D.E. Ong, F. Chytil, Retinoid-binding protein distribution in the developing mammalian nervous system, Development 109 (1990) 75–80.
[46] W. Wurst, A.B. Auerbach, A.L. Joyne, Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum, Development 120 (7) (1994) 2065–2075.
[47] A. Schmidt-Rhaesa, S. Harzsch, G. Purschke, Structure and Evolution of Invertebrate Nervous Systems, Oxford university press, 2016, p. 776.
[48] S. Galler, J. Litzlbauer, M. Kröss, H. Grassberger, The highly efficient holding function of the mollusc 'catch' muscle is not based on decelerated myosin head cross-bridge cycles, Proc. Biol. Sci. 277 (1682) (2010) 803–808.
[49] V.L. Trainer, B.D. Bill, Characterization of a domoic acid binding site from Pacific razor clam, Aquat. Toxicol. 69 (2004) 125–132.
[50] D. Tran, E. Fournier, G. Durrieu, J.C. Massabuau, Inorganic mercury detection by valve closure response in the freshwater clam *Corbicula fluminea*: integration of time and water metal concentration changes, Environ. Toxicol. Chem. 26 (7) (2007) 1545–1551.
[51] E. Grosling, Bivalve Molluscs Biology, Ecology and Culture, Fishing New Books, Blackwell Science, 2004.
[52] E. Grosling, Marine Bivalve Molluscs, second ed., Wiley-Blackwell, 2015.
[53] N.L. Cooper, J.R. Bidwell, Cholinesterase inhibition and impacts on behavior of the Asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide, Aquat. Toxicol. 76 (2006) 258–267.
[54] S. Pillen, I.M. Arts, M.J. Zwarts, Muscle ultrasound in neuromuscular disorders, Muscle Nerve 37 (6) (2008) 679–693.
[55] X. de Montaudouin, M. Lucia, C. Binias, M. Lassudrie, M. Baudrimont, A. Legeay, N. Raymond, F. Jude-Lemeilleur, C. Lambert, N. Le Goïc, F. Garabetian, P. Gonzalez, H. Hégaret, P. Lassus, W. Mehdioub, L. Bourasseau, G. Daffe, I. Paul-Pont, M. Plus, V.T. Do, G. Meisterhans, N. Mesmer-Dudons, N. Caill-Milly, F. Sanchez, P. Soudant, Why is Asari (=Manila) clam Ruditapes philippinarum fitness poor in Arcachon Bay: a meta-analysis to answer? Estuar. Coast Shelf Sci. 179 (2016) 226–235.