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

F. Pierron, P. Gonzalez, A. Bertucci, C. Binias, E. Mérour, Michel Brémont,
X. de Montaudouin

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1 **Title: Transcriptome-wide analysis of wild Asari (=Manila) clams affected by the Brown**
2 **Muscle Disease: etiology and impacts of the disease**

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4
5 **Authors:** Pierron F.¹, Gonzalez P.¹, Bertucci A.¹, Binias C.¹, Mérour E.², Brémont M.², de
6 Montaudouin X.¹

7
8
9 **Full postal addresses**

10 ¹Univ. Bordeaux, CNRS, EPOC 5805, Talence, France.

11 ²Unité de Virologie et Immunologie Moléculaires, INRA, CRJ, Jouy en Josas, France

12
13
14 **Corresponding author: Fabien PIERRON**

15 Address : Université de Bordeaux, Place du Docteur Bertrand Peyneau, 33120 Arcachon,
16 France

17 Tel: +33 556 223 921

18 mail: fabien.pierron@u-bordeaux.fr

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26 **Abstract:**

27

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

47

48

49 **Key words:** RNA-Seq, brown muscle disease, *Venerupis phillipinarum*, neurotoxicity.

50 **1. Introduction**

51

52 Asari (=Manila) clam (*Ruditapes philippinarum*) is a worldwide exploited bivalve native
53 from Indo-Pacific region (Flassch and Leborgne 1992, de Montaudouin et al. 2016, Chiesa et
54 al. 2017). China declares 98% of the world production with a steady yearly ca. 3 % increase
55 (FAO 2014). The cumulative production of other countries is decreasing since 1983, from 200
56 kt to less than 80000 t in 2014. This alarming statistics is mainly due to native countries (except
57 China) like Japan (-88% since 1983). However, in a more recent period, the situation is also
58 preoccupying in countries where Asari clams were introduced like in Canada (50% drop
59 between 2012 and 2014) or Italy (5% decrease between 20007 and 2014 (FAO 2014)). During
60 the last International Symposium on Asari clam in 2015, at Tsu (Japan), concerned countries
61 tried to identify the different reasons explaining this negative trend in the production (Watanabe
62 and Higano 2016). Although very local (or national) factors could be identified, there was a
63 common agreement to point out that in general infectious diseases are a major cause of
64 mortality. This is consistent with the general idea that infectious diseases represent the first
65 cause of mortality in aquaculture (Carnegie 2005), although in the case of Asari clam sources
66 of production can be aquaculture and/or fishing.

67 The most commonly recorded pathogens of Asari clam are the prokaryotic *Vibrio tapetis*
68 (Paillard et al. 2006) and the alveolate *Perkinsus olseni* (Soudant et al. 2013, Ruano and Batista
69 2015), although some metazoans can locally impact clam populations like the pycnogonid sea
70 spider *Nymphonella tapetis* (Toba et al. 2016, Tomiyama et al. 2016) and trematode species
71 (Endo and Hoshina 1974, Dang et al. 2009a). Few years ago, a pathology named Brown Muscle
72 Disease (BMD) was described in Arcachon Bay (Dang et al. 2008), the most productive French
73 area for Asari clam (50% of capture) (de Montaudouin et al. 2016). BMD symptoms were
74 exhaustively described, the main macroscopic feature being the necroses of the posterior
75 adductor muscle (Dang et al. 2008, Dang and de Montaudouin 2009). A questioning point was

76 the lack of impact on the anterior adductor muscle which presents roughly the same histologic
77 organization (Dang et al. 2009b). Besides, the propagation of the necrosis does not seem to be
78 random but to develop from the striated muscle part of the muscle to rapidly invade the totality
79 of the muscle (thus including smooth muscle part) (Dang et al. 2008). BMD had also heavy
80 consequences on the general fitness of the clam, with significant negative effect on the Asari
81 clam index condition (flesh weight versus shell weight ratio) (Dang and de Montaudouin 2009,
82 Dang et al. 2009b, Binias et al. 2014). The research of the etiological agent of BMD privileged
83 a viral infection based on histology and transmission electron microscopy observations (Dang
84 et al. 2009c). All infected clams exhibited electron-dense particles of 25 to 35 nm in all tissues,
85 in contrast with healthy clams in which no virus-like particles were detected. These virus-like
86 particles were isolated, but however we were unable to experimentally infect clams
87 (unpublished data). Clinical and histological observations were different from the few previous
88 studies describing viral or viral-like infections in adults of *Ruditapes* spp. Clams (Novoa and
89 Figueras 2000, Arcangeli et al. 2012, Bateman et al. 2012, Volpe et al. 2017).

90 In the present study, in order to get more insights on the etiology and impacts of the BMD
91 on clams, we used a large scale without a priori RNA-Seq based approach. More specifically,
92 RNA-Seq approach was used to (i) test whether viral RNA sequences can be specifically found
93 in the transcriptome of diseased clams (Arzul et al., 2017) and (ii) identify the genes, and by
94 extension, biological functions that are differentially regulated between diseased and healthy
95 clams. The muscle transcriptome of specimens was determined by high throughput RNA
96 sequencing using Illumina HiSeq 2000 technology. From these results, a new sampling was
97 carried out to collect diseased and healthy animals but also animals with no visible sign of
98 BMD and located at the surface of the sediment. The transcription level of a reduced number
99 of genes previously highlighted by the RNA-Seq study and involved in synaptic transmission
100 was determined by quantitative RT-PCR.

101

102 2. Material and methods

103

104

105 2.1 Wild clam sample collection

106 For RNA-Seq analyses, specimens of *V. philippinarum* were collected from an intertidal
107 site (44°41'N, 01°04'W) of the Arcachon Bay, which characteristics were described in **Binias**
108 **et al. (2014)**. Manila clams were sampled in autumn 2012. Two following status were sampled:
109 buried (BUR) animals with no sign (-) of Brown Muscle Disease BMD (BUR⁽⁻⁾) and clams at
110 the surface (SURF) of the sediment exhibiting signs (+) of BMD (SURF⁽⁺⁾). Each sampled clam
111 was opened with a scalpel and BMD occurrence was estimated by eye. BMD intensity was
112 assessed through the Muscle Print Index (MPI). MPI characterizes the percentage of the
113 posterior adductor muscle surface colonized by the brown muscle on a scale of 0 to 4 as follows:
114 0 (healthy = without BMD), 1 (0-25% of the muscle surface is affected), 2 (25-50%), 3 (50-
115 75%) and 4 (75-100%) (**Dang et al., 2008**). Two BUR⁽⁻⁾ clams (MPI = 0, length = 30 mm) and
116 three SURF⁽⁺⁾ clams (MPI = 3, length = 32 mm) were immediately dissected. Posterior adductor
117 muscle were immediately fixed in RNAlater solution and stored at -80 °C until needed for
118 analyses.

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

124

125 2.2 Preparation of cDNA libraries, contig assembly and RNA-Seq data analyses

126 Samples of posterior adductor muscle were homogenized by means of a bead mill
127 homogenizer using ceramic beads (40 sec, MP Biomedicals) in 500 µl of Trizol reagent. Total
128 RNAs were extracted using the RNeasy Mini kit (Qiagen). During this step, samples were
129 submitted to DNaseI treatment, according to the manufacturer's instructions. A total of 5 clams
130 were used, i.e. 1 pool of 3 SURF⁽⁺⁾ clams and 1 pool of 2 BUR⁽⁻⁾ clams. Then, preparation of
131 cDNA libraries for Illumina HiSeq 2000 sequencing was done using the Truseq RNA sample
132 preparation v2 kit (Illumina), following the manufacturer's instructions. The two individually
133 tagged libraries (one library per pool, BUR⁽⁻⁾ and SURF⁽⁺⁾) were pooled in equal amounts and
134 sequenced on 1 lane at the Genome and Transcriptome Platform of Toulouse (Genotoul,
135 France) using Illumina HiSeq 2000 technology (100 bp paired-ends reads).

136 Base-calling was performed using the ng6 processing environment (Mariette et al.,
137 2012). Sequence quality was checked using the Burrows-Wheeler Aligner and fastQC software
138 (Li and Durbin, 2009). *De novo* assembling was carried out using the Oases software (Schulz
139 et al., 2012) and the Velvet algorithm (Zerbino and Birney, 2008). Chimeric sequences and
140 sequences with a length inferior to 200 bp were discarded. To annotate the contigs based on
141 similarity with known proteins, contigs were blasted on the nr protein database using BLAST
142 program. Gene transcription level was normalized by using RPKM (Reads Per Kilobase per
143 Million mapped reads; Mortazavi et al. 2008) before functional analyses.

144 Functional classification and assessment of significant differential representation of
145 functional classes were performed with the Blast2go software (Conesa et al. 2005) using Gene
146 Ontology annotation and the Fisher's exact test (enrichment analysis). To do this a reference
147 list of genes was constructed. Repetitive contigs (i.e. multiple contigs that had the same
148 annotation), non-annotated contigs or contigs with low homology (Evalue > 10⁻¹⁰) were
149 discarded. A total of 8414 unique genes of known function were identified and were used as a
150 reference. From this reference list, the test lists were constituted by the genes that were down-

151 regulated (fold change < 0.2) and/or up-regulated (fold change > 5) in diseased (SURF⁽⁺⁾)
152 compared to healthy (BUR⁽⁻⁾) clams.

153

154 2.3 Quantitative RT-PCR analyses

155

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

162 For each sample, RNA quality was evaluated by electrophoresis on a 1% agarose gel and
163 concentrations as well as purity were determined by spectrophotometry (Take3, Epoch,
164 Biotek). First-strand cDNA was synthesized from total RNA using the GoScript Reverse
165 Transcription System (Promega), according to the manufacturer's instructions. Following the
166 reverse transcriptase reaction, cDNA was diluted 10-fold. Real-time PCR reactions were then
167 performed in an MX3000P (Stratagene; 95 °C for 10 min, followed by 40 cycles of 95 °C for
168 15 s and 60 °C for 30 s and 72°C for 30 s). Each 20 µL reaction contained 12.5 µL of GoTaq
169 qPCR master mix (Promega), 5 µL template and the specific primer pairs at a final
170 concentration of 250 nM each. Specific primer pairs were designed by means of the
171 Primer3plus software (Table S1). The reaction specificity was determined for each reaction by
172 gel electrophoresis and from the dissociation curve of the PCR product. This was obtained by
173 following the SyberGreen fluorescence level during a gradual heating of the PCR products from
174 60 to 95 °C. Amplification efficiencies for all primer sets were calculated; all values proved to
175 be sufficient to allow direct comparison of amplification plots according to the $\Delta\Delta C_t$ method

176 (Livak and Schmittgen, 2001). Relative quantification of gene expression was achieved by
177 concurrent amplification of the *cilia- and flagella-associated protein 20* gene (*cfap20*) and the
178 *cleavage and polyadenylation specificity factor subunit 5* gene (*nutd21*). Indeed, the
179 transcription of these two genes was found to be unaffected by BMD in the previous RNA-
180 analysis (i.e. their fold change between SURF⁽⁺⁾ and BUR⁽⁻⁾ clams was equal to 1). The gene
181 *nutd21* was found to be the most relevant under our conditions and was finally used as
182 endogenous control.

183

184 2.4 Statistical analyses

185 Comparisons among clams groups were performed by analysis of variance (ANOVA),
186 after checking assumptions of normality (Kolmogorov-Smirnov) and homoscedasticity of the
187 error terms (Levene). When the assumptions were not met as deduced from ad-hoc tests, we
188 used box-cox data transformations or the nonparametric Kruskal–Wallis test. If significant
189 effects were detected, the Least Square Deviation (LSD) or U-Mann–Whitney tests were used
190 to determine whether means between pairs of samples were significantly different from one
191 another. Computations were performed using STATISTICA version 6.1 software (StatSoft)
192 and XLSTAT (Addinsoft version 2012.6.08). Numerical results are given as means \pm SE.

193

194 3. Results

195

196 3.1 RNA-Seq data

197 RNA-seq generated 342 million fragments averaging 75 bases in length. The assembly
198 of these reads generated a total of 47,339 contigs, with a mean size of 2,392 bp (N50 = 3105).
199 A total of 15,849 contigs showed homology with known sequences (BLASTX, Evalue $\leq 10^{-10}$).
200 Finally, a total of 8,414 unique genes of known function were identified (i.e. multiple

201 contigs that had the same annotation were removed and only the hit with the best Evalue was
202 retained for each gene).

203

204 3.2 Viral sequences

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

211

212 3.3 Impacts of the BMD on clam's transcriptome

213 Analyses were carried out to identify the genes that were differentially regulated (by at
214 least a factor 5) between diseased (SURF⁽⁺⁾) and healthy animals (BUR⁽⁻⁾). A total of 358 unique
215 genes were identified, with 206 genes being up-regulated (Table S2) and 152 genes being
216 down-regulated (Table S3) in SURF⁽⁺⁾ compared to BUR⁽⁻⁾ clams. An enrichment analysis with
217 Fisher's exact test ($p < 0.01$) was performed on these differentially expressed genes to highlight
218 the most significant biological processes that differed between the two groups of animals (Fig.
219 2). The principal functions represented among the 358 differentially transcribed genes are
220 summarized in Fig. 2A. Concerning the biological processes and molecular functions
221 associated to the down-regulated genes in diseased animals (Fig. 2B), the synaptic transmission
222 process (GO terms: regulation of excitatory postsynaptic membrane potential, negative
223 regulation of synaptic transmission, learning, cholinesterase activity, terminal bouton and in a
224 lesser extent cellular calcium homeostasis) was the most represented. It is noteworthy that this
225 process was the only one that was highlighted at a more stringent threshold ($FDR < 0.05$, see

226 **Fig. 3**). No significant result was obtained at this threshold with the list of up-regulated genes.
227 The other down-regulated functions were related to the inflammatory response (GO terms: cell
228 migration, response to glucocorticoid stimulus), to blood coagulation (GO terms: positive
229 regulation of blood coagulation, fibrinolysis) and to cell differentiation and division (GO terms:
230 positive regulation of ERK1 and ERK2 cascade, tyrosine metabolic process, skeletal muscle
231 fiber development). Biological processes and molecular functions associated to the up-
232 regulated genes in diseased animals were related to the immune response (GO terms:
233 embryonic hemopoiesis, retinoic acid metabolic process), oxidative stress (GO term:
234 glutathione peroxidase activity), development and morphogenesis (GO terms: embryonic
235 forelimb morphogenesis, retinoic acid metabolic process) and cellular amide metabolic process.
236

237 3.4 Impacts of the BMD on genes involved in synaptic transmission

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

249

250 4. Discussion

251 Numerous viruses belonging to different families (*Herpesviridae*, *Papovaviridae*,
252 *Togaviridae* for example) have been previously reported as infecting marine molluscs (Meyer
253 et al., 2009, Arzul et al., 2017). Most of them were identified in cultivated organisms for
254 aquaculture like oysters, mussels, scallops or abalones. However, there is still a lack of
255 molecular information concerning these viruses, mainly due to their identification which is
256 classically based on histological studies. Indeed, few marine viral genomes have been
257 completely characterized and most of the time only those having important economic impact
258 have been extensively studied. This is the case, for example, of the *Herpesviridae* OsHV-1
259 which is worldwide associated with high mortality outbreaks in several marine species
260 including the Pacific oyster *Magallana* (= *Crassostrea*) *gigas* (Renault and Novoa, 2004,
261 Vásquez-Yeomans et al., 2010). New generation sequencing, using high throughput approaches
262 like RNA-Seq, has started revealing the huge diversity of marine viruses and has been
263 evidenced to be a useful tool to identify and characterize virus sequences in their host (Arzul
264 et al, 2017, Brum et al, 2015, Martínez Martínez et al, 2014). In the current study we have
265 identified one contig, among all the contigs assembled from the derived-RNAseq sequences,
266 which presented extensive homologies with retroviruses genes *rt-ltr*, *rnase-H* and *rve*. These
267 retrovirus-related sequences were present only in infected clams. Retroviruses genomes encode
268 structural and enzymatic viral proteins. They are usually organized as: 5'LTR-*gag-pol-env*-
269 3'LTR (Leblanc et al, 2013; Balvay et al, 2007). In these genomes *gag* encodes for the
270 nucleocapsid protein and *env* for the viral envelope glycoprotein, while the *pol* gene encodes
271 for a reverse transcriptase, a RNase-H and an integrase proteins. These extensive homologies
272 with the POL polyproteins and the fact that this fragment has been only encountered in diseased
273 clams suggest that the RPHIL_POL2.4.16 fragment could represent a part of the genome from
274 the etiologic agent of the BMD. This finding is consistent with previous description of the BMD
275 agent where virus like particles (VLPs) of around 30 nm have been evidenced (Dang et al,

276 2009c). Based on size, structure and cellular position authors suggested that these VLPs could
277 belong to the picorna-like family. Our finding strengthened this hypothesis since such viruses
278 possess single RNA genomes and belong to retro-like viruses. However, future prospects using
279 molecular approaches will be necessary to unequivocally relate this virus to a family.

280 Concerning the impacts of the BMD on the muscle transcriptome of clams, according
281 to our previous findings at both the transcriptional or histological levels (Dang et al., 2008 and
282 2009c; Binias et al., 2014), we found an up-regulation of genes involved in the defense against
283 oxidative stress (GO term: glutathione peroxidase activity with the genes: glutathione
284 peroxidase 1, fold change (FC) 11.1; chorion peroxidase, FC 11.5; glutathione peroxidase 2,
285 FC 13.1;). In the same way, we observed significant changes in the transcription levels of
286 inflammatory- and immune-related genes. Genes involved in the immune or inflammatory
287 response (Figure 1) were both up- and down-regulated between SURF⁽⁺⁾ and BUR⁽⁻⁾ clams. For
288 example, among the most down-regulated genes, we found two genes encoding for proteins
289 that are two major actors of the innate immunity, the component complement C3 (FC 0.07) and
290 the big defensin 3 (FC 0.1) (Brogden, 2005; Song et al., 2010). Among the most up-regulated
291 genes, we found a gene involved in the inflammation (the gene tyrosine-protein kinase Lck, FC
292 12.1; Gaeste et al., 2009) and two other genes known to be involved in the host defense during
293 pathogen infection (the gene pathogenesis-related thaumatin-like protein 1, FC 11.5 and the
294 gene tandem repeat galectin, FC 11.8; Allam et al., 2014; Petre et al., 2011). Similar findings
295 were also reported in diverse bivalve species infected by bacteria or protozoans (see Allam et
296 al., 2014). For authors, some transcripts are up-regulated while others are switched down
297 providing a tailored response to the pathogen (Allam et al., 2014). An alternative hypothesis in
298 our case could be that the BMD agent deregulates the clam defense. Indeed, the persistence of
299 a virus in a host depends on its ability to evade and/or deregulate the host defense. Numerous
300 viral mechanisms of immune evasion were described in order to establish virus persistence,

301 including a down-regulation of complement C3 mRNA transcription (Stoermer and Morrison
302 2011; Mazumdar et al., 2012). We also found that several genes involved in skeletal muscle
303 fiber development were down-regulated. This is in accordance with that fact that the BMD
304 triggers posterior adductor muscle atrophy (Dang et al., 2008).

305 Most surprisingly, among the biological processes that were affected in diseased
306 animals in comparison to healthy animals, the synaptic transmission process was the most
307 represented. Genes involved in synaptic transmission (e.g. cholinesterase precursor (*bche*),
308 cholinesterase isoform x2, glutamate receptor kainate 2-like (*grik2*), serine threonine-protein
309 phosphatase 2b catalytic subunit alpha isoform isoform 2 (*ppp3ca*), serum response factor,
310 ankyrin isoform u) were down-regulated. Moreover, the down-regulated gene FRMFamide (FC
311 0.1) encodes for a neuropeptide that have been proven to be especially useful to visualize the
312 developing nervous system in molluscs (Dyachuk and Odintsova, 2009). We must note
313 however that one gene involved in synaptic transmission was found to be up-regulated (FC
314 6.4), the gene encoding for the 5-hydroxytryptamine receptor 2a (*htr2a*). Moreover, among the
315 up-regulated functions identified, several could be associated to nervous system development.
316 For example, in the class retinoic acid metabolic process, two up-regulated genes, i.e. cellular
317 retinoic acid-binding protein 1 and 2 (FC 10.1 and 12.5, respectively), were found to be
318 involved in the development and differentiation of the mammalian nervous system by
319 interacting with certain homeobox genes (Maden et al., 1990). Moreover, in the class
320 embryonic forelimb morphogenesis, the up-regulated gene homeobox protein engrailed-1 (FC
321 6) was found to be involved in the development of the central nervous system in mammals
322 (Wurst et al., 1994). In bivalves, the nervous system is made up by three pairs of ganglia, the
323 cerebropleural ganglia, the pedal ganglia and the visceral ganglia. The visceral ganglia are
324 located on the surface of the posterior adductor muscle. Visceral ganglia not only control the
325 posterior adductor muscle but also the posterior foot retractor muscles, gills, heart pericardium,

326 kidney, the posterior region of the digestive tract, gonad, the mantle, siphons and pallial sense
327 organs. In contrast to the posterior muscle, the anterior adductor muscle does not present
328 ganglia on its surface but is innervated by nervous projections from the cerebropleural ganglia
329 (Schmidt-Rhaesa et al. 2015). It is noteworthy that the BMD only affects the posterior adductor
330 muscle of animals, leading to its atrophy. The anterior muscle was never found to be affected
331 in both diseased and healthy animals (Dang et al., 2008, 2009c). Transcriptomic data coupled
332 with previous anatomopathological findings could suggest that the nervous system and notably
333 visceral ganglia are a main cellular target of the BMD pathogen. Among the deregulated genes
334 previously evoked, several are known to be involved in the control of muscle contraction or
335 relaxation. The up-regulated gene *htr2a* encodes for a receptor for serotonin. Serotonin is
336 involved in muscle relaxation and valve opening in bivalves (Galler et al., 2010). Three genes
337 encoding for proteins involved in the metabolism/pathway of excitatory neurotransmitters
338 glutamate and acetylcholine (i.e. *bche*, *grik2* and in a lesser extent *ppp3ca*) were down-
339 regulated in diseased animals (Galler et al., 2010; Trainer and Bill, 2004). Cholinesterase is the
340 enzyme responsible for the inactivation of acetylcholine, a neurotransmitter known to trigger
341 muscle adductor contraction and subsequently valves closure in bivalves (Galler et al., 2010).
342 Valve closure is indeed an active mechanism while opening is achieved passively by ligaments.
343 Valve closure is required to protect animals from predators or contaminants (Tran et al. 2007),
344 to expulse pseudofaeces, but also in clams, in association with the foot and siphons, to bury
345 (Grosling, 2004 and 2015). We previously reported a reducing ability of BMD clams to bury
346 (Dang et al., 2008). In the case of BMD animals, this could be linked to the atrophy of posterior
347 adductor muscle. However, although *V. philippinarum* typically lives buried in the sediment,
348 BMD clams are always found at the surface of the sediment in association with other Asari
349 clams with no visible sign of BMD (SURF⁽⁻⁾ clams). In our previous works, we found that
350 SURF⁽⁻⁾ animals presented intermediate transcription level of genes involved in immune

351 response, in mitochondrial metabolism or the oxidative stress response as well as intermediate
352 phagocytosis capacity and intermediate condition index between BUR⁽⁻⁾ and SURF⁽⁺⁾ clams,
353 suggesting that muscle atrophy (i.e. BMD) could be a latter sign of the pathology (Binias et al.,
354 2014). Taken together, these results could suggest that the nervous system and more
355 specifically neuromuscular junctions could be a primary site of action of the BMD agent. In
356 support of this hypothesis, in a study carried out on the freshwater bivalve *Corbicula Fluminea*,
357 Cooper and Bidwell (2006) have shown that cholinesterase inhibition reduces the capacity of
358 animals to burrow into the substrate. Moreover, muscle atrophy and calcification,
359 anatomopathological characteristics of the BMD (Dang et al., 2009c), are known post-
360 syndromes of neuromuscular disorders (Pillen et al., 2008).

361 In order to gain a deeper insight into such hypothesis, we came back in the field (year
362 2016) to collect BUR⁽⁻⁾, SURF⁽⁻⁾ and SURF⁽⁺⁾ animals. We then determined the gene
363 transcription level of *bche*, *grik2* and *ppp3ca* by quantitative RT-PCR in the posterior adductor
364 muscle of individuals. According to previous results obtained by RNA-Seq in BUR⁽⁻⁾ and
365 SURF⁽⁺⁾ individuals collected in year 2012, the transcription level of *bche*, *grik2* and *ppp3ca*
366 was found to be significantly down-regulated in SURF⁽⁺⁾ clams in comparison to BUR⁽⁻⁾ clams.
367 In addition, SURF⁽⁻⁾ clams presented intermediate values between BUR⁽⁻⁾ and SURF⁽⁺⁾ animals.
368 These findings reinforce the hypothesis that muscle atrophy (i.e. BMD) is a latter sign of the
369 pathology and that nervous system could be instead a primary target of the BMD agent.
370 Interestingly, we previously reported the presence of free unenveloped virus-like particles
371 (VLPs) in the cytoplasm of rectal cells of BMD clams, suggesting direct penetration. It is
372 intriguing to speculate that the infectious agent penetrates *via* the digestive system before
373 affecting motor neurons, leading *in fine*, and perhaps only in some cases, to muscle atrophy.
374 Such a mode of action could be comparable to that of the poliovirus in humans (Singh et al.,
375 2013).

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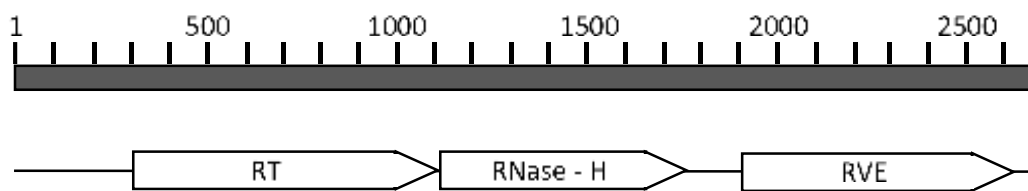
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Figures

Figure 1

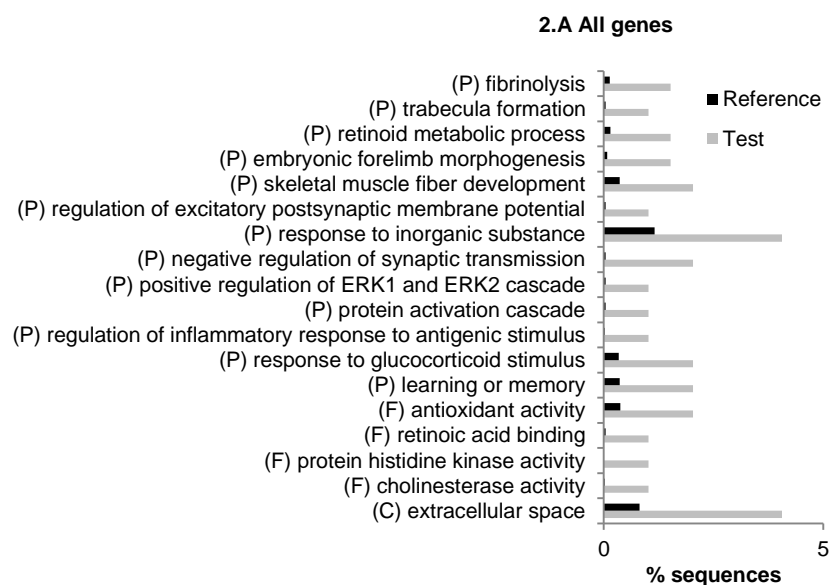
Proteins encoded by the RPHIL_POL2.4.16 contig showing homologies with retroviruses *pol* gene products. RT = Reverse transcriptase; RVE = Retrovirus integrase



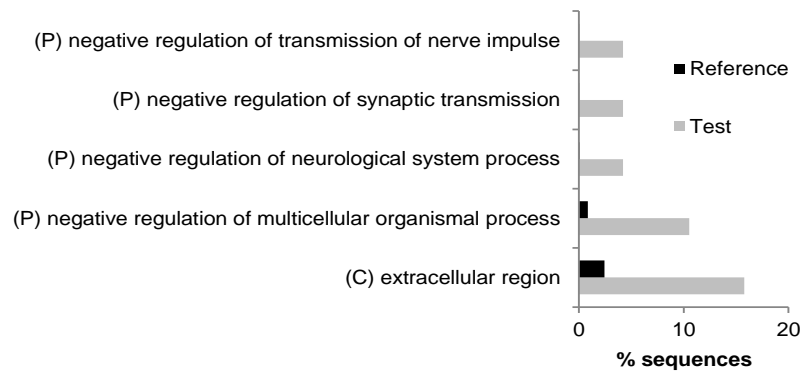
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Figure 2

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).



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583 Figure 3

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

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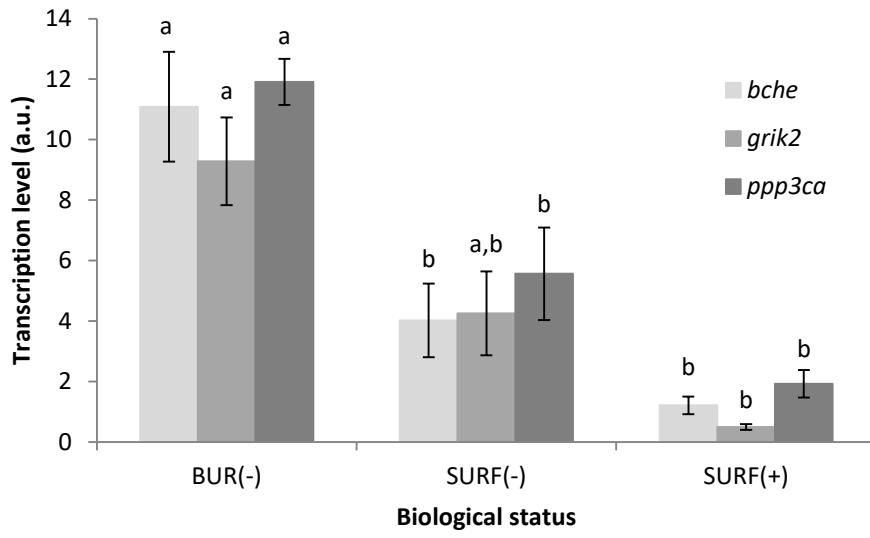
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603 Figure 4

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605 Change in the transcription levels (mean \pm SE; n = 10) of *bche*, *grik2* and *ppp3ca* in the
606 posterior adductor muscle of clams collected in spring 2016 and presenting different biological
607 status (BUR⁽⁻⁾, SURF⁽⁻⁾, SURF⁽⁺⁾). Bars sharing same-case letters do not differ significantly (P
608 > 0.05).

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