

Metabolic responses of clam *Ruditapes philippinarum* exposed to its pathogen *Vibrio tapetis* in relation to diet

Richard Gaëlle ^{1,*}, Guérard Fabienne ¹, Corporeau Charlotte ², Lambert Christophe ¹,
Paillard Christine ¹, Pernet Fabrice ²

¹ UMR 6539 CNRS UBO IRD IFREMER, LEMAR – IUEM – UBO, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest-Iroise – Rue Dumont d'Urville, 29280 Plouzané, France

² Ifremer, UMR 6539 LEMAR (CNRS/UBO/IRD/Ifremer), Technopôle Brest-Iroise CS 10070, 29280 Plouzané, France

* Corresponding author : Gaëlle Richard, email address : gaelle.richard@univ-brest.fr

Abstract :

We investigated the effect of brown ring disease (BRD)¹ development and algal diet on energy reserves and activity of enzymes related to energy metabolism, antioxidant system and immunity in Manila clam, *Ruditapes philippinarum*. We found that algal diet did not impact the metabolic response of clams exposed to *Vibrio tapetis*. At two days post-injection (dpi), activities of superoxide dismutase and glutathione peroxidase (GPx) decreased whereas activities of nitric oxide synthase (iNOS) and catalase increased in infected clams, although no clinical signs were visible (BRD-). At 7 dpi, activities of several antioxidant and immune-related enzymes were markedly increased in BRD-likely indicating an efficient reactive oxygen species (ROS) scavenging compared to animals which developed clinical signs of BRD (BRD+). Therefore, resistance to BRD clinical signs appearance was associated with higher detoxification of ROS and enhancement of immune response. This study provides new biochemical indicators of disease resistance and a more comprehensive view of the global antioxidant response of clam to BRD development.

Highlights

► Brown ring disease affects antioxidant and immune response of clams. ► Resistance to the clam disease is associated with higher antioxidant activities. ► New bio-indicators of resistance to brown ring disease development are provided.

Keywords : Antioxidant enzymes, immunity, energetic metabolism, *Ruditapes philippinarum*, *Vibrio tapetis*

List of abbreviations

- BRD, brown ring diseases;
- dpi, days post injection;
- ROS, reactive oxygen species;
- RNS, reactive nitrogen species;
- t-SOD, total superoxide dismutase;
- Cu/Zn-SOD, Cu/Zn superoxide dismutase;
- Mn-SOD, Mn superoxide dismutase;
- CAT, catalase;
- t-GPx, total glutathion peroxidase;
- Se-GPx, selenium-dependant glutathione peroxidase;
- GR, glutathion reductase;
- GST, glutathione-S-transferase;
- TrxP, thioredoxin peroxidase;
- TrxR, thioredoxin reductase;
- iNOS, inducible nitric oxide synthase;
- PO, phenoloxidase;
- HK, hexokinase;
- PK, pyruvate kinase;
- CS, citrate synthase

1. Introduction

The bacteria *Vibrio tapetis* is the causative agent of Brown Ring Disease (BRD) which has affected the Manila clam *Ruditapes philippinarum* worldwide since the late 80's (Paillard and Maes, 1994). The main clinical sign of BRD is a brown organic deposit called conchiolin on the inner shell of the clam. This defense response of the mantle is called "nacrezation" and involves the deposition of conchiolin to enclose the pathogen followed by the deposition of new calcified layers which may lead to shell repair (Paillard and Maes, 1995; Trinkler et al., 2010). When these defense responses are not sufficient, lesions occur and the pathogen invades the host leading to septicaemia and death (Allam et al., 2002).

Bacterial exposure can induce important alterations in the energy reserves of bivalves and consequently on their metabolism. For instance, clams infected with *V. tapetis* exhibit glycogen reserve depletion, weight loss and lower condition indices (Gouletquer and Université de Bretagne Occidentale, 1989; Plana, 1995; Plana et al., 1996). Also, energetic status of the host plays a major role in the outcome (death or recovery) of a bacterial infection in bivalves (Paillard, 2004; Flye-Sainte-Marie et al., 2007; Genard et al., 2013). For instance, oyster *Crassostrea virginica* larvae exposed to *Vibrio corallilyticus* show a marked decline in food intake which coincides with lower triglyceride and protein content, the two main energetic reserves in bivalve larvae (Genard et al., 2013).

The invertebrate immune system is non-adaptive or innate and incorporates cellular and humoral components (Ellis et al., 2011). The main cellular defense mechanism relies on phagocytosis by immunocompetent cells known as haemocytes, and it consists of several steps including recognition, chemotaxis, attachment, incorporation and destruction through

54 the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Pipe
55 and Coles, 1995; Chu, 2000; Soudant et al., 2013; Donaghy et al., 2015).

56 The internal destruction of pathogenic agents in bivalves relies on an increased
57 production of ROS and RNS that are toxic to invaders (Donaghy et al., 2015). Accumulation of
58 ROS and RNS can result in oxidative stress in the host (Lesser, 2006), which is normally
59 prevented by antioxidant enzymes (Sies, 1993; Hermes-Lima, 2004). Superoxide dismutase
60 (SOD) transforms the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) (Manduzio et
61 al., 2005). H_2O_2 is decomposed into H_2O and O_2 by catalase (CAT) or by glutathione
62 peroxidases (GPx) and thioredoxin peroxidase (TrxP) when H_2O_2 and organic peroxides
63 concentration are low (Hermes-Lima, 2004; Manduzio et al., 2005). Activities of GPx and TrxP
64 depend on oxidation states of glutathione and thioredoxin (Trx) respectively, which are
65 controlled by glutathione reductase (GR) and thioredoxin reductase (TrxR) (Powis and
66 Montfort, 2001). Finally, glutathione-S-transferase (GST) is an antioxidant enzyme involved
67 in xenobiotics detoxification and scavenging of lipid peroxides (Hermes-Lima, 2004;
68 Manduzio et al., 2005). These reactions are summarized in Figure 1.

69 Many studies have investigated the effect of bacterial infection on gene expression of
70 antioxidant enzymes in bivalves, but very few consider effects on enzymatic activity.
71 Expression of *RpSOD*, *RpGST* and *RpTrx* increased in gills and haemocytes of *R. philippinarum*
72 during a short-term exposure to *V. tapetis* (Revathy et al., 2012a, 2012b; Umasuthan et al.,
73 2012a, 2012b, 2012c). Although gene expression of antioxidant enzymes is sometimes
74 correlated with their activity (Genard et al., 2013), this is not always the case (Chen et al.,
75 2002; Canesi et al., 2010; Béguel et al., 2013). Activities of SOD, CAT and GST increased in
76 response to bacterial infection whereas other enzymes were not influenced or not examined
77 (Canesi et al., 2010; Genard et al., 2013; Le Bris et al., 2015).

78 In addition to antioxidant enzymes, other immune-related enzymes are involved in
79 pathogen destruction in bivalves. Inducible nitric oxide synthase (iNOS) produces nitric oxide
80 (NO), a RNS which putatively leads to the formation of peroxynitrite, nitrosothiols and other
81 nitrogen derivative in cells that are harmful to pathogens (Hausladen and Stamler, 1999;
82 Donaghy et al., 2015). Additionally, *in vitro* production of nitrites, which are NO breakdown
83 products, is correlated with the concentration of *V. tapetis* in hemolymph and extrapallial

84 fluids of *R. philippinarum* (Jeffroy and Paillard, 2011). To our knowledge, iNOS activity has
85 never been investigated in bivalves during exposure to pathogen.

86 Finally, phenoloxidases (PO) have a key role in innate immunity in bivalves by
87 activating the melanization cascade which leads to the production of melanin and its
88 derivatives (Aladaileh et al., 2007; Cerenius et al., 2008). These compounds exhibit
89 fungistatic, bacteriostatic and antiviral properties (Cerenius et al., 2008; Luna-Acosta et al.,
90 2011). PO is a particularly important aspect of BRD as it contributes to the conchiolin deposit
91 formation (Paillard et al., 1994). Bivalves exposed to pathogens generally exhibit higher PO
92 activity than control animals (Butt and Raftos, 2008; Le Bris et al., 2015; Richard et al., 2015).

93 In bivalves, haemocyte functions are influenced by dietary fatty acids (Delaporte et
94 al., 2003, 2006, 2007; Dudognon et al., 2014). We investigated the effect of diet on the
95 dynamics of BRD in *R. philippinarum* in relation to reproductive status in another paper
96 (Richard, 2015). We showed that disease prevalence and intensity as well as haemocyte
97 parameters were not influenced by diet. However, sexual maturity enhanced BRD intensity,
98 likely reflecting an energetic trade-off between reproduction and immunity.

99 Objectives of the present study were to investigate the effect of BRD development
100 and algal diet quality during a *V. tapetis* exposure on: (1) lipid, protein and carbohydrate
101 content, and activity of enzymes related to energy metabolism such as hexokinase (HK),
102 pyruvate kinase (PK) and citrate synthase (CS); (2) activity of the antioxidant enzymes SOD,
103 CAT, GPx, (GR), (TrxR), and GST, and (3) activity of two immune-related enzymes, iNOS and
104 PO. In this study, mantle of clams was individually sampled and pooled for biochemical
105 analysis according to diet, injection, tank replicate and BRD status at each time post-
106 injection (2, 7 and 30 dpi). The originality of our work is that we clearly describe the
107 metabolic response of clams exposed to *V. tapetis* as a function of disease development. In
108 particular, we characterize the metabolic response associated with the appearance of
109 disease clinical signs, thereby providing new biochemical indicators of disease resistance.
110 Also, we obtain a more comprehensive view of the global antioxidant response of clam to
111 the disease than previous studies which have generally focused only on SOD and CAT.

112 2. Material and methods

113 2.1. Experimental design

114 Animals and experimental design are fully described in a companion paper (See
115 Richard, 2015). Briefly, clams were acclimated for four weeks with either *Isochrysis aff.*
116 *galbana*, clone Tahitian (T-Iso) or *Chaetoceros calcitrans*, two algal species exhibiting
117 differences in their biochemical compositions. Then, clams were injected in extrapallial
118 cavity with 100 μ L of CECT4600^T *V. tapetis* suspension (10^7 UFC.mL⁻¹) or 100 μ L of filtered-sea
119 water (FSW) and further monitored for 30 days. Clam shells were collected at 2, 7 and 30 dpi
120 to assess disease prevalence intensity. Additionally, sex and sexual maturity were evaluated
121 and tested as internal parameters influencing BRD development.

122 Algal cultures of *C. calcitrans* and T-Iso were collected three times during the course
123 of the conditioning period for fatty acid composition analyses. Briefly, algae were filtered on
124 pre-ignited GF/F filters, placed into 6 mL vials containing 3 mL of chloroform:methanol (2:1,
125 v:v) and stored at -20°C until analyses.

126 Clams injected with *V. tapetis* and control animals injected with FSW collected at 2, 7
127 and 30 dpi were carefully dissected on ice. Whole clam mantle (Ma) and digestive gland (DG)
128 were flash-frozen in liquid nitrogen and stored at -80°C. Samples were individually powdered
129 using a Retsch MM 400 under liquid nitrogen. Then, an equal amount of powder originating
130 from the same condition of diet \times injection treatment \times BRD status \times tank for each organ at
131 each sampling time were pooled together to obtain enough biological materials for
132 biochemical analysis, such that each individual had the same weight contribution to the pool
133 (Table 1). Clams injected with *V. tapetis* were classified as either BRD- (no clinical sign of
134 infection) or BRD+ (visible brown ring). For the control, visibly healthy clams were selected.
135 Only mature male were analyzed because the sex ratio was in favor of males (1.5:1.0) and
136 most of them were mature. Each pool consisted of 2 to 4 individuals per condition. The
137 number of replicates was therefore not homogenous because it depends on clinical signs
138 development and reproduction stages occurrence.

139 Ma were analyzed for carbohydrate and protein contents, and immunity-,
140 antioxidant- and metabolism-related enzyme activities at 2, 7 and 30 dpi. Immune related
141 enzymes were analyzed through iNOS and PO activities, antioxidant enzymes through SOD,
142 CAT, GPx, TrxR, GR, GST activities and metabolism-related enzymes through citrate synthase
143 (CS), HK and pyruvate kinase (PK) activities.

144 Additionally, Ma samples were analyzed for fatty acid composition of polar lipids at 7
145 dpi to evaluate peroxidation index in membrane lipids. Fatty acids of neutral lipids were
146 analyzed in DG at 2 dpi to verify that diet effectively influenced the biochemical composition
147 of clams. Indeed, fatty acids composition of neutral lipids generally mirrors that of the food
148 (Dalsgaard et al., 2003). For these analyses, powder of DG and Ma was transferred into 6mL
149 vials containing chloroform:methanol mixture (2:1, v:v) and stored at -20°C until analyses.

150 **2.2. Biochemical analyses**

151 **2.2.1. Fatty acid**

152 Fatty acid composition was analyzed in total lipid of algae, in neutral lipids of clam DG
153 at 2 dpi and in polar lipids of clam Ma at 7 dpi. Neutral and polar lipids were separated using
154 a silica gel micro-column as described in Marty et al. (Marty et al., 1992). Briefly, aliquots of
155 samples were evaporated until dry and lipid extracts were recovered after three
156 chloroform:methanol (98:2, v/v) washings. The resulting extracts were placed on top of a
157 silica gel micro-column. Columns were washed with 10 mL chloroform:methanol (98:2, v/v)
158 to elute neutral lipids followed by 15 mL of methanol to elute polar lipids. Then, a known
159 amount of tricosanoic acid (23:0) was added as an internal standard in both fractions. Each
160 lipid fraction was transesterified using 10% boron-trifluoride methanol (Metcalf and
161 Schmitz, 1961) and analyzed in a gas chromatograph with an on-column injector, DB-Wax
162 capillary column and a flame ionization detector. Fatty acids were then identified by
163 comparison of retention times with standards and checked using a non-polar column.

164

165

166 2.2.2. Carbohydrate

167 Carbohydrates contents were analyzed using the method of Dubois et al. (Dubois et
168 al., 1956). Carbohydrates were extracted from Ma in a 10 mM phosphate buffered saline
169 (PBS) containing EDTA (1mM) and Triton® X-100 (0.1%) at the ratio of 50 mg of powder in
170 250 µL of buffer. Standard solution of 0.4 mg/mL glucose was serially diluted in PBS (ranging
171 from 0.4 to 0.01 mg/mL) to provide a standard curve for the assay. Then, 500µL of 5%
172 phenol solution was added to 250 µL of samples and standard solutions. The resulting
173 solution was allowed to incubate during 40 min in the dark. The colorimetric reaction was
174 initiated by addition of 2.5 mL H₂SO₄. After 40 min, all solutions were placed in cuvettes and
175 absorbance was read at 490 and 600 nm on an Uvikon 941 (Kontron instruments).
176 Carbohydrate content was calculated using a standard curve and the following expression:

$$OD_s = OD_{s,490} - 1.5 \times (OD_{s,600} - 0.003)$$

177 Where OD_s is the sample optical density of the sample; $OD_{s,490}$ is the sample optical density
178 at 490 nm; and $OD_{s,600}$ is the sample optical density at 600 nm. Results are expressed as mg
179 per g wet weight.

180 2.2.3. Protein

181 For each sample, 50 mg of powder was homogenized in 250µL of extraction buffer
182 using an Ultra-Turrax®. Two extraction buffers were tested and used for enzymatic assays in
183 order to avoid interactions with assays buffers and therefore with enzymatic activities. The
184 extraction buffer used prior to SOD, PO, PK, HK, CS, GPx-t and GPx-Se enzymatic assays
185 consisted in NaCl (150mM), Tris HCl (10mM), EDTA (1mM), EGTA (1mM), phosphatase
186 inhibitor cocktail II (1%; Sigma-Aldrich), Triton® X-100 (1%; Sigma-Aldrich), CA-630 Igepal®
187 (0.5%; Sigma-Aldrich) and 1 tablet/25mL of complete EDTA free protease inhibitor cocktail
188 (Roche) (Corporeau et al., 2012). The extraction buffer used prior to CAT, GR, GST, NOS and
189 TrXR enzymatic assays consisted in Phosphate buffered saline (PBS 10mM; Sigma-Aldrich),
190 EDTA (1mM) and Triton® X-100 (0.1%; Sigma-Aldrich). After homogenization, samples were
191 centrifuged at 1000g for 10 minutes at 4°C to eliminate the lipid fraction of the samples and
192 then centrifuged at 10000g for 45 minutes at 4°C. The resulting supernatants were split

193 among 9-10 separate microtubes for subsequent protein and enzyme assays, and stored at -
194 80°C.

195 Protein concentrations were evaluated in samples previously homogenized with each
196 buffer by the Bradford method using the Biorad Protein Assay Dye Reagent Concentrate
197 (BioRad France) and Bovine Serum Albumin (BSA) as protein standard. Results are expressed
198 as mg per g wet weight.

199 **2.2.4. Enzyme**

200 Activities were measured using 96-well microplates (Greiner 96-F-bottom) and
201 Synergy HT (Bio-Tek), except for catalase activity which was measured using quartz cuvettes
202 and Uvikon 941 (Kontron instruments).

203 **Energy-related enzymes**

204 Citrate Synthase (CS, EC 2.3.3.8) was assayed by recording the conversion of DTNB
205 into TNB (Childress and Somero, 1979). Briefly, 20 µL of protein supernatant were added to
206 triplicate wells as well as serial dilutions of commercial CS from porcine heart (Sigma-Aldrich
207 France) (ranging from 5 to 100 mU/mL), which was used as a standard for this reaction. One
208 hundred and sixty µL of assay buffer (0.25 mM acetyl-CoA, 0.125 mM DTNB and 100 mM
209 Tris-HCl) were added to wells and the reaction was initiated by addition of 20 µL of 5 mM
210 oxaloacetate. In blank wells, samples were replaced by extraction buffer. Absorbance was
211 monitored for 10 min at 412 nm. Results are expressed in U/mg protein; one U being defined
212 as the amount of enzyme catalyzing 1 µmole of TNB per min ($\epsilon_{\text{TNB}, 412} = 13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

213 Pyruvate Kinase (PK, EC 2.7.1.40) and hexokinase (HK, EC 2.7.1.1) activities were
214 measured using the protocol developed by Greenway and Storey (Greenway and Storey,
215 1999). Twenty µL of protein supernatant were loaded in triplicate wells and 20 µL of
216 extraction buffer was used for the blank wells. For PK, 180 µL of assay buffer (100 mM
217 imidazole-HCl, 50 mM KCl, 5 mM MgCl₂, 10 mM phosphoenolpyruvate, 2 mM NADH, 0.2%
218 (v/v) rotenone saturated ethanol and 1 U/mL lactate dehydrogenase) were added to the
219 wells and the reaction was monitored for 15 min at 340 nm. For HK, the reaction was
220 initiated by addition of 200 µL of assay buffer (100 mM Tris, 1 mM EDTA, 2 mM MgCl₂, 5 mM

221 glucose, 1 mM ATP, 0.2 mM NADP⁺ and 1 U/mL Glucose-6-Phosphate Dehydrogenase) and
222 reaction kinetics was followed during 15 min at 340 nm. Results are expressed as U/mg
223 protein; one U being defined as the amount of enzyme oxidizing 1 μ mole of NADH or NADP⁺
224 (using $\epsilon_{\text{NADH or NADP}^+, 340} = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

225 **Antioxidant enzymes**

226 Total Superoxide Dismutase (t-SOD, EC 1.15.1.1) activity was measured using the SOD
227 Assay Kit (Sigma-Aldrich, France) following a modified procedure (Richard et al., 2015).
228 Briefly, the inhibition of the xanthine/xanthine oxidase complex was followed recording the
229 absorbance at 440 nm after 20 minutes of incubation at 25°C. Additionally, mitochondrial
230 SOD (Mn SOD) activity was assayed by addition of potassium cyanide, which was found to
231 inhibit specifically cytosolic SOD (Cu-Zn SOD) at concentrations above 2mM in *Mytilus edulis*
232 (Manduzio et al., 2003). In a preliminary assay, we tested a range of potassium cyanide
233 concentrations (0 – 10 mM) and the 5 mM concentration was selected for the Mn SOD assay
234 in clams. Mn SOD activity and subsequently Cu-Zn SOD activity (calculated as the activity
235 difference between t-SOD and Mn SOD) were assayed using the same kit and adding 5 mM
236 potassium cyanide to the assay buffer. All SOD activities are expressed in U/mg total protein
237 where one unit is the amount of enzyme necessary for inhibiting by 50% the
238 xanthine/xanthine oxidase complex.

239 Catalase (CAT, EC 1.11.1.6) activity was assessed following Aebi (Aebi, 1984). Briefly,
240 8 μ L of protein supernatant were added to 792 μ L of hydrogen peroxide solution (10 mM) to
241 initiate the reaction. Absorbance was immediately recorded for 90 sec at 240 nm. The blank
242 consisted of extraction buffer instead of sample. Absorbance of the blank was subtracted
243 from that of the samples. Activities are expressed in U/mg protein where 1 U is the amount
244 of enzyme necessary for catalyzing 1 μ mole of H₂O₂ per min (using $\epsilon_{\text{H}_2\text{O}_2, 240} = 0.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).
245

246 Total Glutathione Peroxidase and Selenium Glutathione Peroxidase (t-GPx and Se-
247 GPx, EC 1.11.1.9) activities were measured by monitoring the decrease in NADPH
248 concentration at 340 nm (McFarland et al., 1999). The assay buffer consisted of 62.5 mM
249 Phosphate Buffer, 6.25 mM EDTA, 2.5 mM GSH, 2.5 U/mL GR, 0.5 mM NADPH. For t-GPx,
250 Cumene Hydroperoxide (1.875mM) was added to the assay buffer. For Se-GPx, NaN₃ (1.25

251 mM) and H₂O₂ (0.3125 mM) were added. Fifty µL of protein supernatants were placed in the
252 wells and the reaction was started by the addition of 200 µL of the assay buffer. NADPH loss
253 was recorded for 3 min at 25°C. The blank consisted of extraction buffer instead of sample.
254 Absorbance of the blank was subtracted from that of the samples. Enzyme activities are
255 reported as U/mg protein where one unit is the amount of enzyme necessary for oxidizing 1
256 µmole of NADPH per min (using $\epsilon_{\text{NADPH}, 340} = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

257 Glutathione Reductase (GR, EC 1.8.1.7) was assessed following (Cribb et al., 1989).
258 Briefly, 20 µL of protein supernatants were loaded in triplicate wells and the reaction was
259 initiated by addition of 170 µL of assay buffer (100 mM Sodium Phosphate Buffer, 0.7 mM
260 NADPH, 0.09 mM DTNB, 0.2 mM GSSG). Kinetics of GR activity was assayed during 3 min at
261 412 nm. The blank consisted of extraction buffer instead of sample. Absorbance of the blank
262 was subtracted from that of the samples. Activities are expressed as U/mg protein; one unit
263 being defined as the amount of enzyme catalyzing 1 µmole of DTNB per min (using $\epsilon_{\text{DTNB}, 412}$
264 $= 14.15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

265 Glutathione-S-Transferase (GST, EC 2.5.1.18) was measured following (McFarland et al.,
266 1999). Briefly, 15 µL of protein supernatant or extraction buffer (for blanks) were placed in
267 triplicate wells. The reaction was initiated by the addition of 200 µL of assay buffer (200 mM
268 Phosphate Buffer, 1 mM CDNB, 1 mM GSH) and absorbance was recorded for 3 min at 340
269 nm. Absorbance of the blank was subtracted from that of the samples. GST activities are
270 reported as U/mg protein; one U being defined as the amount of enzyme catalyzing 1 µmole
271 of CDNB per min (using $\epsilon_{\text{CDNB}, 340} = 9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

272 Thioredoxin Reductase (TrxR, EC 1.8.1.9) was assessed following the conversion of
273 DTNB into TNB (Smith and Levander, 2002). Briefly, 50 µL of protein supernatant were
274 loaded in triplicate wells and 200 µL of assay buffer (62.5 mM Phosphate Buffer, 12.5 mM
275 EDTA, 0.3 mM NADPH and 3.125 M DTNB) were added to initiate the reaction. Additionally,
276 each sample was assayed in the presence of 20 µM aurothioglucose (an inhibitor of TrxR), in
277 order to assess non-TrxR activity contributions. After one minute of incubation at room
278 temperature, kinetics were followed for 3 min at 412 nm. The blank consisted of extraction
279 buffer instead of sample. Absorbance of the blank was subtracted from that of the samples.

280 Activities are expressed as U/mg protein; one U being defined as the amount of enzyme
281 producing 1 μ mole of TNB per min ($\epsilon_{\text{TNB}, 412} = 13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

282

283 Immune related enzymes

284 For the Inducible Nitric Oxide Synthase activity (iNOS, EC 1.14.13.39) assay, we
285 developed a non-radioactive method (appendix A). Briefly, 55 μ L of protein supernatants
286 were incubated for 30 minutes at 37°C with 5 μ L of assay buffer: Tris buffer (20 mM pH 7.4),
287 NADPH (1 mM), L-Arginine (1 mM), 5,6,7,8-tetrahydrobiopterin (10 μ M), FAD+ (5 μ M).
288 Samples and blanks were also incubated with the same assay buffer containing L-NMMA
289 (200 μ M), a specific inhibitor of NOS, in order to assess NOS activity alone. Blanks were run
290 (PBS buffer instead of samples) and iNOS from mouse was used as a standard for the range
291 curve. The reactions were stopped by addition of 200 μ L of the color developing reagent
292 (COLDER, see description in appendix A) and the microplate was incubated at 95°C for 15
293 minutes. After 10 minutes at room temperature, L-citrulline formation was assessed by
294 reading absorbance at 530 nm. Absorbance of the blank and of samples incubated with L-
295 NMMA was subtracted from that of the samples. Activities are expressed in U/mg protein
296 where 1 U is the amount of enzyme necessary to produce 1 μ mole of L-citrulline per min.

297 Phenoloxidase activity was assessed according to (Le Bris et al., 2013). Briefly, 50 μ L
298 of protein supernatants or extraction buffer (blank) were incubated with 50 μ L of Tris-HCl
299 buffer (0.10 M, pH 8.0) for 10 minutes at 25°C. The reaction was initiated by addition of 100
300 μ L 0.04 M L-3,4-dihydroxyphenylalanine (L-DOPA), a common substrate for the three PO
301 subclasses. The increase in absorbance due to L-DOPA oxidation was monitored for 30
302 minutes at 492 nm. The spontaneous oxidation of L-DOPA (blank) was also measured and
303 subtracted from samples values. PO specific activities are expressed in units per mg protein
304 using the following expression:

$$PO (U/mg) = (\Delta OD/min \times f_{d,s})/[protein]$$

305 Where $\Delta OD/min$ is the value of increment of optical density per minute; $f_{d,s}$ is the dilution
306 factor applied to the sample; and $[protein]$ is the protein concentration of the sample in
307 mg/mL.

308 2.3. Statistical analyses

309 Given the unbalanced nature of the sampling design, injection, BRD status and
310 sampling time were merged into one explanatory variable, hereafter referred to as
311 "treatment". Treatments consist of seven conditions: control at 2 dpi, BRD- at 2dpi, control
312 at 7 dpi, BRD- at 7 dpi, BRD+ at 7 dpi, control at 30 dpi and BRD+ at 30 dpi.

313 Non parametric Permutational Multivariate Analyses of Variance (PERMANOVA,
314 Anderson, 2001) were conducted on all biochemical variables for Ma and DG. This method
315 allows partitioning the variance in response variables attributed to each explanatory variable
316 (diet and treatment) and their interactions. A pseudo F-ratio, defined as the ratio of
317 dissimilarity within a treatment and between treatments, is computed and its significance
318 was tested by 999 permutations.

319 Ordination analyses were then performed using Principal Component Analysis (PCA)
320 to plot individuals, variables and correlations between variables. A *posteriori* comparison
321 tests were performed for each variable (ANOVA) in order to estimate the significance of
322 results obtained with PERMANOVA. These analyses investigated the effect of diet, treatment
323 and their interaction on biochemical parameters of clams. Analyses were first conducted
324 with data collected at all sampling times and secondly for each sampling time.

325 Statistical analyses were carried out using R 3.1.2 Software (R Development Core
326 Team, 2011). PERMANOVA were achieved using *adonis* function and the homogeneity of
327 multivariate dispersions were tested using *betadisper* function from the *vegan* package
328 (Oksanen et al., 2015). The *rda* function from *vegan* package was used for PCAs analyses and
329 the subsequent biplots were drawn using *ggplot2* package (Wickham and Chang, 2015).
330 ANOVA using type III error were performed using the *Anova* function from the *car* package
331 (Fox and Weisberg, 2010).

332

333

334 **3. Results**

335 **3.1. Dietary fatty acid signatures**

336 The two cultured algae exhibited typical fatty acid profiles: *C. calcitrans* is rich in
337 20:5n-3 and 20:4n-6 and poor in 22:6n-3 whereas T-*Iso* is rich in 22:6n-3, deficient in 20:5n-3
338 and poor in 20:4n-6 (Table 2). Neutral lipids of clam DG at the end of the conditioning period
339 clearly mirrored the fatty acid composition of the diet. For instance, DG of clams fed *C.*
340 *calcitrans* were rich in 20:4n-6 and 20:5n-3 and poor in 22:6n-3 while this is the inverse
341 pattern for those fed T-*Iso* (Table 3).

342 However, polar lipids of clam Ma at 7 dpi were much less influenced by the diet.
343 Indeed, levels of 20:4n-6, 20:5n-3, 22:6n-3, in polar lipids of animals fed *C. calcitrans* were
344 5.4%, 7.4%, and 19.5% compared to 5.0%, 5.7%, and 21.6% in those fed T-*Iso*, suggesting
345 that selective incorporation/elimination of dietary fatty acids has occurred in this organ.

346 **3.2. Metabolic response of clam**

347 Overall, proximate composition (lipid, protein and carbohydrate content) and
348 activities of enzymes related to energy, antioxidant and immunity in Ma of clams were not
349 influenced by diet. However, most of these variables were greatly influenced by treatment
350 (PERMANOVA, $r^2=0.30$, $F=2.19$, $p=0.029$).

351 At 2 and 7 dpi, activities of t-SOD, CAT, Se-GPx, GR, GST, iNOS and PO were markedly
352 influenced by the interaction of diet x treatment (PERMANOVA, $r^2=0.28$, $F=4.08$, $p<0.01$).
353 However, the effect of diet was not significant when PCAs and PERMANOVAs are conducted
354 by day (Table 4). We therefore consider only treatment effects.

355 Principal component analysis allows discrimination of clams injected with *V. tapetis*
356 from control at 2 dpi (Figure 2). At 7 dpi, PCA discriminates clams injected with *V. tapetis*
357 which did not exhibit clinical signs of disease development (BRD-) from those which were
358 visibly unhealthy (BRD+) and control. Activities of t-SOD, CAT, Se-GPx, GR and PO were
359 correlated together along with positive values of the first principal component axis (PC1)
360 whereas proximate compositions were correlated together along with PC1 negative values.

361 Activities of Cu/Zn-SOD and TrxR were respectively correlated with negative and with
362 positive values of the second principal component (PC2).

363 At 2 dpi, activities of Cu/Zn-SOD and CAT were not significantly influenced by
364 injection x BRD status, although they were respectively 23 % lower and 27 % higher in BRD-
365 than in control (Figure 3, Appendix B). Also, activity of Se-GPx in BRD- was 46 % lower than
366 that of control. Finally, activity of iNOS was 24 % higher in BRD- than that of control.

367 At 7 dpi, activities of antioxidant enzymes (t-SOD, CAT, GR) and PO in BRD- were 20 to
368 40 % higher than those recorded in other animals, regardless of sampling times and BRD
369 status (Figure 3, Appendix B). Activity of Se-GPx in BRD+ was 54 % lower than that of BRD- at 7 dpi
370 (Figure 3, Appendix B). Activity of GST was not significantly impacted by treatment at 7 dpi,
371 although it was 29 % lower in BRD+ than in control and BRD- (Appendix B). Finally, activity of
372 iNOS in BRD+ was 30 % higher than that of control at 2 and 7 dpi and BRD- at 7 dpi (Figure 3,
373 Appendix B).

374 At 30 dpi, principal component analysis (PCA) and PERMANOVA by sampling times showed
375 that there was no significant effect at this sampling time (Table 4).

376

377 **4. Discussion**

378 **4.1. Diet effect on clam response to *V. tapetis***

379 The two culture algae exhibited typical fatty acid profiles which were mirrored in the
380 neutral lipids of the DG as previously reported in other bivalve species (Delaunay et al.,
381 1993; Soudant et al., 1996, 1999). However, polar lipids of clam MA at 7 dpi were much less
382 influenced by the diet, suggesting that selective incorporation of dietary fatty acids has
383 occurred in this organ. Although selective incorporation of essential fatty acids in polar lipids
384 is common in bivalves (Delaunay et al., 1993; Soudant et al., 1996; Copeman et al., 2002;
385 Pernet and Tremblay, 2004), this phenomenon has rarely been observed to such an extent.
386 For instance, polar lipids of clam haemocytes are reflective of the diet and clearly influence
387 their functions (Delaporte et al., 2003, 2006, 2007; Dudognon et al., 2014). Our result may

388 then reflect differences between organs abilities to regulate PUFAs in polar lipids and/or
389 differences related to batch origin (Napolitano and Ackman, 1992; Soudant et al., 1999;
390 Pernet et al., 2008).

391 Finally, the fact that dietary deficiencies were compensated in clam mantle may
392 explain that disease prevalence and intensities, as well as activities of immunity-,
393 antioxidant- and energy metabolism-related enzymes were not influenced by diet in this
394 organ.

395 **4.2. Metabolic response of clam to *V. tapetis***

396 Activities of antioxidant and immune-related enzymes of clams injected with *V.*
397 *tapetis* changed between 2 dpi and 7 dpi concomitantly with increasing prevalence of BRD
398 and changes in BRD status (Richard, 2015). In our study, biochemical parameters were
399 measured in mature males only. Therefore, our results may not be fully representative of
400 clam populations which generally consist of a mixture of males and females at different
401 reproductive stages. However, the effects of sex and reproductive stage on enzyme activity
402 in bivalves have received very little attention. The only available information is that sex and
403 reproductive investment do not influence activities of antioxidant enzymes in gills of oysters
404 (Béguel et al., 2013).

405 **4.2.1. Early response at 2 dpi**

406 In *V. tapetis* injected clams, activity of Cu/Zn-SOD tends to decrease (-23 %) whereas
407 activity of iNOS was significantly higher than in the control (+24 %), likely leading to higher
408 concentration of $O_2^{\bullet-}$ and NO in Ma of BRD-. Indeed, previous studies reported that
409 productions of ROS and RNS are enhanced to neutralize pathogens in bivalves (Arumugan et
410 al., 2000; Tafalla et al., 2002; Bugge et al., 2007; Villamil et al., 2007; Costa et al., 2008). In
411 particular, the enhancement of iNOS in BRD- agrees with an *in vitro* analysis of body fluids of
412 clams exposed to *V. tapetis* that showed higher levels of nitrates and nitrites, two
413 breakdown products of NO decomposition as compared to unexposed controls (Jeffroy and
414 Paillard, 2011). Finally, increasing concentrations of both $O_2^{\bullet-}$ and NO might lead to the
415 formation of peroxynitrite (ONOO-), a RNS highly toxic to pathogen (Torreilles and Guerin,
416 1999; Donaghy et al., 2015).

417 Activity of Se-GPx, an enzyme involved in peroxides decomposition, was markedly
418 lowered in BRD- (-46 % compared to control), suggesting ROS accumulation in Ma. However,
419 this effect might be counterbalanced by the activity of CAT, an enzyme also involved in
420 peroxide detoxification, which tended to increase in BRD- (+27 % compared to control). The
421 complementary role of CAT and GPx has already been observed in clams *Meretrix meretrix*
422 challenged with *Vibrio parahaemolyticus* (Wang et al., 2011, 2013).

423 **4.2.2. Late response at 7 dpi**

424 **Resistance to BRD clinical signs appearance is associated to higher detoxication of ROS and** 425 **PO activities**

426 Activities of several antioxidant enzymes (t-SOD, CAT) were markedly increased in
427 BRD-, compared to BRD+ and control, at 2 and 7 dpi (respectively, +22 % and +41 %). Over-
428 activation of these antioxidant enzymes in BRD- coincided with an increase in GR activity
429 (+22 % compared to control and BRD+ at 2 and 7 dpi), likely reflecting the regulation of
430 glutathione redox status for its further use, both in Se-GPx functioning and also as a non-
431 enzymatic antioxidant (Winston and Digiulio, 1991; Hermes-Lima, 2004). Overall, the
432 enhancement of antioxidant pathways in BRD- could indicate an efficient ROS scavenging
433 compared to BRD+. These results showed that over-activation of antioxidant enzymes in
434 clams may thus be related to their resistance to BRD clinical signs appearance. Although
435 increasing antioxidant enzymes activities during early exposure of bivalves to vibriosis was
436 already reported (Canesi et al., 2010; Genard et al., 2013), this phenomenon has never been
437 related to the resistance to BRD clinical signs appearance.

438 Concomitantly, the activity of PO was 31 % higher in BRD- compared to that of BRD+
439 and control at 2 and 7 dpi. Interestingly, oysters *Saccostrea glomerata* selectively bred for
440 QX disease resistance exhibit a similar increase in PO activity compared to wild animals (Butt
441 and Raftos, 2008). It is therefore likely that increased PO activity in clams exposed to *V.*
442 *tapetis* is related to a resistance to BRD clinical signs appearance as previously suggested for
443 antioxidant enzymes.

444 Taken together, these results suggest that in *V. tapetis*-challenged clams at 7 dpi, a
445 resistance to BRD clinical signs appearance can be measured and was associated with

446 detoxification of ROS by antioxidant enzymes and the production of toxic quinones through
447 PO.

448 **Appearance of BRD clinical signs is associated with lower GST activities and higher iNOS** 449 **activities**

450 The activity of GST, an enzyme associated with xenobiotics and lipid peroxide
451 detoxification, tended to be lower in BRD+ compared to that of BRD- and control at 7 dpi (-
452 29 %). Concomitantly, t-SOD, CAT and Se-GPx activities of BRD+ were lower than those of
453 BRD- at 7 dpi (respectively, -22 %, -41 % and -54 %). Altogether, our results could suggest
454 that BRD+ were exposed to higher level of peroxidized lipids and ROS at 7 dpi than BRD- as
455 lipid peroxidation increase has previously been associated to decreased antioxidant activities
456 in *M. edulis* (Viarengo et al., 1991; Power and Sheehan, 1996). However, unsaturation index
457 of polar lipids, an indicator of lipid peroxidation (Hulbert, 2003; Munro and Blier, 2012), was
458 similar in BRD-, BRD+ and control at 7 dpi, such that the reduction in GST and antioxidant
459 activities in BRD+ was not sufficient to alter the polar lipids of clams.

460 In addition, the activity of iNOS was 20% higher in BRD+ compared to that of control
461 clams at 2 and 7 dpi and BRD- at 7 dpi, likely leading to higher levels of NO and RNS in these
462 animals, as previously reported (Jeffroy and Paillard, 2011). Over activation of iNOS had
463 already been reported at 2 dpi as an early response to *V. tapetis* injection, and BRD+ at 7 dpi
464 may keep on producing RNS to fight bacterial infection.

465 **5. Conclusion**

466 Antioxidant and immune responses were strongly influenced by BRD development in
467 clams exposed to *V. tapetis*. An early response was observed at 2 dpi in visibly healthy clams
468 (BRD-). This early response consisted of a decrease in antioxidant activities, likely leading to
469 accumulation of ROS to cope with bacterial infection. Then, at 7 dpi, clams that remained
470 BRD- exhibited an over-activation of PO and antioxidant enzymes compared to diseased
471 animals (BRD+). Therefore, activities of antioxidant and immune related enzymes are
472 biomarkers of resistance to BRD development in clam. This study provides a better
473 understanding of the antioxidant response of clams to the disease and it shows for the first
474 time that iNOS takes part in the *in vivo* response of a bivalve species to its pathogen.

475 **Acknowledgements**

476 This work was supported by the "Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19) and
477 co-funded by a grant from the French government under the program "Investissements
478 d'Avenir" and by a grant from the Regional Council of Brittany. This work was also supported
479 by University of Western Brittany. We would first like to thank F. Fonteneau for providing
480 the clams, C. Quéré for her help with fatty acid analyses, P. Miner for his help with
481 experimental design and maintenance, and A. Bidault, N. Le Goïc, F. Nunes, H. Hégaret, C.
482 Fabioux, I. Paul-Pont, F. Le Grand, F. Riera, A.L. Cassone, P. Le Souchu, A. Muir, E. Harney, Y.
483 Epelboin, M. Fuhrmann, M. Provost, L. Frère, F. Boullot, B. Dubief, M. Czamanski, R.
484 Morvezen, V. Foulon, M. Protat, A. Guyon, and L. Delisle for their help with sampling and O.
485 Gauthier for advices in statistical analysis. Special thanks are addressed to E. Harney for
486 linguistic revision.

487 **References**

- 488 Aebi, H., 1984. Catalase *In vitro*. *Methods Enzymol.* 105, 121–126.
- 489 Aladaileh, S., Nair, S.V., Raftos, D.A., 2007. Induction of phenoloxidase and other
490 immunological activities in Sydney rock oysters challenged with microbial pathogen-
491 associate molecular patterns. *Fish Shellfish Immunol.* 23, 1196–1208.
492 doi:10.1016/j.fsi.2007.05.003
- 493 Allam, B., Paillard, C., Ford, S.E., 2002. Pathogenicity of *Vibrio tapetis*, the etiological agent of
494 brown ring disease in clams. *Dis. Aquat. Organ.* 48, 221–231. doi:10.3354/dao048221
- 495 Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance.
496 *Austral Ecol.* 26, 32–46. doi:10.1046/j.1442-9993.2001.01070.x
- 497 Arumugan, M., Romestand, B., Torreilles, J., 2000. Nitrite released in haemocytes from
498 *Mytilus galloprovincialis*, *Crassostrea gigas* and *Ruditapes decussatus* upon
499 stimulation with phorbol myristate acetate. *Aquat. Living Resour.* 13, 173–177.
500 doi:10.1016/S0990-7440(00)00150-9
- 501 Béguel, J.-P., Huvet, A., Quillien, V., Lambert, C., Fabioux, C., 2013. Study of the antioxidant
502 capacity in gills of the Pacific oyster *Crassostrea gigas* in link with its reproductive
503 investment. *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* 157, 63–71.
504 doi:10.1016/j.cbpc.2012.10.004
- 505 Bugge, D.A., Hegaret, H., Wikfors, G.H., Allam, B., 2007. Oxidative burst in hard clam
506 (*Mercenaria mercenaria*) haemocytes. *Fish Shellfish Immunol.* 23, 188–196.
507 doi:10.1016/j.fsi.2006.10.006
- 508 Butt, D., Raftos, D., 2008. Phenoloxidase-associated cellular defence in the Sydney rock
509 oyster, *Saccostrea glomerata*, provides resistance against QX disease infections. *Dev.*
510 *Comp. Immunol.* 32, 299–306. doi:10.1016/j.dci.2007.06.006
- 511 Canesi, L., Barmo, C., Fabbri, R., Ciacci, C., Vergani, L., Roch, P., Gallo, G., 2010. Effects of
512 *vibrio* challenge on digestive gland biomarkers and antioxidant gene expression in
513 *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* 152, 399–
514 406. doi:10.1016/j.cbpc.2010.06.008
- 515 Cerenius, L., Lee, B.L., Soderhall, K., 2008. The proPO-system: pros and cons for its role in
516 invertebrate immunity. *Trends Immunol.* 29, 263–271. doi:10.1016/j.it.2008.02.009
- 517 Chen, G.A., Gharib, T.G., Huang, C.C., Taylor, J.M.G., Misek, D.E., Kardina, S.L.R., Giordano, T.J.,
518 Iannettoni, M.D., Orringer, M.B., Hanash, S.M., Beer, D.G., 2002. Discordant protein
519 and mRNA expression in lung adenocarcinomas. *Mol. Cell. Proteomics* 1, 304–313.
520 doi:10.1074/mcp.M200008-MCP200
- 521 Childress, J., Somero, G., 1979. Depth-Related Enzymic Activities in Muscle, Brain and Heart
522 of Deep-Living Pelagic Marine Teleosts. *Mar. Biol.* 52, 273–283.
523 doi:10.1007/BF00398141
- 524 Chu, F.L.E., 2000. Defense mechanisms of marine bivalves.
- 525 Copeman, L.A., Parrish, C.C., Brown, J.A., Harel, M., 2002. Effects of docosahexaenoic,
526 eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid
527 composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live
528 food enrichment experiment. *Aquaculture* 210, 285–304. doi:10.1016/S0044-
529 8486(01)00849-3
- 530 Corporeau, C., Vanderplancke, G., Boulais, M., Suquet, M., Quere, C., Boudry, P., Huvet, A.,
531 Madec, S., 2012. Proteomic identification of quality factors for oocytes in the Pacific

- 532 oyster *Crassostrea gigas*. J. Proteomics 75, 5554–5563.
533 doi:10.1016/j.jprot.2012.07.040
- 534 Costa, M.M., Novoa, B., Figueras, A., 2008. Influence of beta-glucans on the immune
535 responses of carpet shell clam (*Ruditapes decussatus*) and Mediterranean mussel
536 (*Mytilus galloprovincialis*). Fish Shellfish Immunol. 24, 498–505.
537 doi:10.1016/j.fsi.2007.10.003
- 538 Cribb, A., Leeder, J., Spielberg, S., 1989. Use of a Microplate Reader in an Assay of
539 Glutathione-Reductase Using 5,5'-Dithiobis(2-Nitrobenzoic Acid). Anal. Biochem. 183,
540 195–196. doi:10.1016/0003-2697(89)90188-7
- 541 Dalsgaard, J., St John, M., Kattner, G., Muller-Navarra, D., Hagen, W., 2003. Fatty acid trophic
542 markers in the pelagic marine environment. Adv. Mar. Biol. Vol 46 46, 225–340.
543 doi:10.1016/S0065-2881(03)46005-7
- 544 Delaporte, M., Soudant, P., Lambert, C., Jegaden, M., Moal, J., Pouvreau, S., Degremont, L.,
545 Boudry, P., Samain, J.-F., 2007. Characterisation of physiological and immunological
546 differences between Pacific oysters (*Crassostrea gigas*) genetically selected for high
547 or low survival to summer mortalities and fed different rations under controlled
548 conditions. J. Exp. Mar. Biol. Ecol. 353, 45–57. doi:10.1016/j.jembe.2007.09.003
- 549 Delaporte, M., Soudant, P., Moal, J., Giudicelli, E., Lambert, C., Segueineau, C., Samain, J.-F.,
550 2006. Impact of 20 : 4n-6 supplementation on the fatty acid composition and
551 hemocyte parameters of the Pacific oyster *Crassostrea gigas*. Lipids 41, 567–576.
552 doi:10.1007/s11745-006-5006-9
- 553 Delaporte, M., Soudant, P., Moal, J., Lambert, C., Quere, C., Miner, P., Choquet, G., Paillard,
554 C., Samain, J.F., 2003. Effect of a mono-specific algal diet on immune functions in two
555 bivalve species - *Crassostrea gigas* and *Ruditapes philippinarum*. J. Exp. Biol. 206,
556 3053–3064. doi:10.1242/jeb.00518
- 557 Delaunay, F., Marty, Y., Moal, J., Samain, J., 1993. The Effect of Monospecific Algal Diets on
558 Growth and Fatty-Acid Composition of *Pecten-Maximus* (l) Larvae. J. Exp. Mar. Biol.
559 Ecol. 173, 163–179. doi:10.1016/0022-0981(93)90051-0
- 560 Development Core Team, R., 2011. R: A Language and Environment for Statistical
561 Computing. Vienna, Austria: the R Foundation for Statistical Computing.
- 562 Donaghy, L., Hong, H.-K., Jauzein, C., Choi, K.-S., 2015. The known and unknown sources of
563 reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs. Fish
564 Shellfish Immunol. 42, 91–97. doi:10.1016/j.fsi.2014.10.030
- 565 Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F., 1956. Colorimetric Method for
566 Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356.
567 doi:10.1021/ac60111a017
- 568 Dudognon, T., Lambert, C., Quere, C., Auffret, M., Soudant, P., Kraffe, E., 2014.
569 Mitochondrial activity, hemocyte parameters and lipid composition modulation by
570 dietary conditioning in the Pacific oyster *Crassostrea gigas*. J. Comp. Physiol. B-
571 Biochem. Syst. Environ. Physiol. 184, 303–317. doi:10.1007/s00360-013-0800-1
- 572 Ellis, R.P., Parry, H., Spicer, J.I., Hutchinson, T.H., Pipe, R.K., Widdicombe, S., 2011.
573 Immunological function in marine invertebrates: Responses to environmental
574 perturbation. Fish Shellfish Immunol. 30, 1209–1222. doi:10.1016/j.fsi.2011.03.017
- 575 Flye-Sainte-Marie, J., Pouvreau, S., Paillard, C., Jean, F., 2007. Impact of Brown Ring Disease
576 on the energy budget of the Manila clam *Ruditapes philippinarum*. J. Exp. Mar. Biol.
577 Ecol. 349, 378–389. doi:10.1016/j.jembe.2007.05.029
- 578 Fox, J., Weisberg, S., 2010. An R Companion to Applied Regression. SAGE.

- 579 Genard, B., Miner, P., Nicolas, J.-L., Moraga, D., Boudry, P., Pernet, F., Tremblay, R., 2013.
580 Integrative Study of Physiological Changes Associated with Bacterial Infection in
581 Pacific Oyster Larvae. *Plos One* 8, e64534. doi:10.1371/journal.pone.0064534
- 582 Gouletquer, P., 1989. Etude des facteurs environnementaux intervenant sur la production
583 de la palourde japonaise d'élevage *Ruditapes philippinarum*. Université de Bretagne
584 occidentale, Brest.
- 585 Greenway, S.C., Storey, K.B., 1999. The effect of prolonged anoxia on enzyme activities in
586 oysters (*Crassostrea virginica*) at different seasons. *J. Exp. Mar. Biol. Ecol.* 242, 259–
587 272. doi:10.1016/S0022-0981(99)00103-3
- 588 Hausladen, A., Stamler, J.S., 1999. Nitrosative stress. *Oxid. Antioxid. Pt B* 300, 389–395.
- 589 Hermes-Lima, M., 2004. Oxygen in Biology and Biochemistry: Role of Free Radicals, in: Ph.D,
590 K.B.S. (Ed.), *Functional Metabolism*. John Wiley & Sons, Inc., pp. 319–368.
- 591 Hulbert, A.J., 2003. Life, death and membrane bilayers. *J. Exp. Biol.* 206, 2303–2311.
- 592 Jeffroy, F., Paillard, C., 2011. Involvement of nitric oxide in the *in vitro* interaction between
593 Manila clam, *Ruditapes philippinarum*, hemocytes and the bacterium *Vibrio tapetis*.
594 *Fish Shellfish Immunol.* 31, 1137–1141. doi:10.1016/j.fsi.2011.10.008
- 595 Le Bris, C., Paillard, C., Stiger-Pouvreau, V., Guerard, F., 2013. Laccase-like activity in the
596 hemolymph of *Venerupis philippinarum*: Characterization and kinetic properties. *Fish*
597 *Shellfish Immunol.* 35, 1804–1812. doi:10.1016/j.fsi.2013.09.009
- 598 Le Bris, C., Richard, G., Paillard, C., Lambert, C., Segueineau, C., Gauthier, O., Pernet, F.,
599 Guerard, F., 2015. Immune responses of phenoloxidase and superoxide dismutase in
600 the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis* - Part I:
601 Spatio-temporal evolution of enzymes' activities post-infection. *Fish Shellfish*
602 *Immunol.* 42, 16–24. doi:10.1016/j.fsi.2014.10.021
- 603 Lesser, M.P., 2006. Oxidative stress in marine environments: Biochemistry and physiological
604 ecology, in: *Annual Review of Physiology*. pp. 253–278.
- 605 Luna-Acosta, A., Saulnier, D., Pommier, M., Haffner, P., De Decker, S., Renault, T., Thomas-
606 Guyon, H., 2011. First evidence of a potential antibacterial activity involving a
607 laccase-type enzyme of the phenoloxidase system in Pacific oyster *Crassostrea gigas*
608 haemocytes. *Fish Shellfish Immunol.* 31, 795–800. doi:10.1016/j.fsi.2011.07.016
- 609 Manduzio, H., Rocher, B., Durand, F., Galap, C., Leboulenger, F., 2005. The point about
610 oxidative stress in molluscs. *ISJ* 2.
- 611 Marty, Y., Delaunay, F., Moal, J., Samain, J., 1992. Changes in the Fatty-Acid Composition of
612 *Pecten Maximus* (I) During Larval Development. *J. Exp. Mar. Biol. Ecol.* 163, 221–234.
613 doi:10.1016/0022-0981(92)90051-B
- 614 McFarland, V.A., Inouye, L.S., Lutz, C.H., Jarvis, A.S., Clarke, J.U., McCant, D.D., 1999.
615 Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown
616 bullhead, *Ameiurus nebulosus*. *Arch. Environ. Contam. Toxicol.* 37, 236–241.
- 617 Metcalfe, L.D., Schmitz, A.A., 1961. The Rapid Preparation of Fatty Acid Esters for Gas
618 Chromatographic Analysis. *Anal. Chem.* 33, 363–364. doi:10.1021/ac60171a016
- 619 Munro, D., Blier, P.U., 2012. The extreme longevity of *Arctica islandica* is associated with
620 increased peroxidation resistance in mitochondrial membranes. *Aging Cell* 11, 845–
621 855.
- 622 Napolitano, G., Ackman, R., 1992. Anatomical Distributions and Temporal Variations of Lipid
623 Classes in Sea Scallops *Placopecten Magellanicus* (gmelin) from Georges Bank (nova-
624 Scotia). *Comp. Biochem. Physiol. B-Biochem. Mol. Biol.* 103, 645–650.
625 doi:10.1016/0305-0491(92)90384-4

- 626 Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L.,
627 Solymos, P., Stevens, M.H.H., Wagner, H., 2015. vegan: Community Ecology Package.
- 628 Paillard, C., 2004. A short-review of brown ring disease, a vibriosis affecting clams, *Ruditapes*
629 *philippinarum* and *Ruditapes decussatus*. Aquat. Living Resour. 17, 467–475.
630 doi:10.1051/alr:2004053
- 631 Paillard, C., Maes, P., 1995. The Brown Ring Disease in the Manila Clam, *Ruditapes*
632 *Philippinarum* .1. Ultrastructural Alterations of the Periostracal Lamina. J. Invertebr.
633 Pathol. 65, 91–100. doi:10.1006/jipa.1995.1015
- 634 Paillard, C., Maes, P., 1994. Brown Ring Disease in the Manila Clam *Ruditapes Philippinarum* -
635 Establishment of a Classification-System. Dis. Aquat. Organ. 19, 137–146.
636 doi:10.3354/dao019137
- 637 Paillard, C., Maes, P., Oubella, R., 1994. Brown ring disease in clams. Annu. Rev. Fish Dis. 4,
638 219–240.
- 639 Pernet, F., Tremblay, R., 2004. Effect of varying levels of dietary essential fatty acid during
640 early ontogeny of the sea scallop *Placopecten magellanicus*. J. Exp. Mar. Biol. Ecol.
641 310, 73–86. doi:10.1016/j.jembe.2004.04.001
- 642 Pernet, F., Tremblay, R., Redjah, I., Sevigny, J.-M., Gionet, C., 2008. Physiological and
643 biochemical traits correlate with differences in growth rate and temperature
644 adaptation among groups of the eastern oyster *Crassostrea virginica*. J. Exp. Biol.
645 211, 969–977. doi:10.1242/jeb.014639
- 646 Pipe, R., Coles, J., 1995. Environmental Contaminants Influencing Immune Function in
647 Marine Bivalve Mollusks. Fish Shellfish Immunol. 5, 581–595. doi:10.1016/S1050-
648 4648(95)80043-3
- 649 Plana, S., 1995. Perturbations de la glande digestive et du métabolisme chez la palourde
650 aquacole, *Ruditapes Philippinarum*, affectée par la maladie de l'anneau brun (PhD
651 thesis). Université de Bretagne occidentale, France.
- 652 Plana, S., Sinquin, G., Maes, P., Paillard, C., Le Pennec, M., 1996. Variations in biochemical
653 composition of juvenile *Ruditapes philippinarum* infected by a *Vibrio sp.* Dis. Aquat.
654 Organ. 24, 205–213. doi:10.3354/dao024205
- 655 Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill
656 and digestive gland of the blue mussel, *Mytilus edulis*. Comp. Biochem. Physiol. C-
657 Pharmacol. Toxicol. Endocrinol. 114, 99–103. doi:10.1016/0742-8413(96)00024-2
- 658 Powis, G., Montfort, W.R., 2001. Properties and biological activities of thioredoxins. Annu.
659 Rev. Biophys. Biomol. Struct. 30, 421–455. doi:10.1146/annurev.biophys.30.1.421
- 660 Revathy, K.S., Umasuthan, N., Lee, Y., Choi, C.Y., Whang, I., Lee, J., 2012a. First molluscan
661 theta-class Glutathione S-Transferase: Identification, cloning, characterization and
662 transcriptional analysis post immune challenges. Comp. Biochem. Physiol. B-Biochem.
663 Mol. Biol. 162, 10–23. doi:10.1016/j.cbpb.2012.02.004
- 664 Revathy, K.S., Umasuthan, N., Lee, Y., Whang, I., Kim, H.C., Lee, J., 2012b. Cytosolic
665 thioredoxin from *Ruditapes philippinarum*: Molecular cloning, characterization,
666 expression and DNA protection activity of the recombinant protein. Dev. Comp.
667 Immunol. 36, 85–92. doi:10.1016/j.dci.2011.06.006
- 668 Richard, G., 2015. Approche mécanistique de la réponse de la palourde japonaise, *Ruditapes*
669 *philippinarum*, exposée à la bactérie *Vibrio tapetis* : influence de la température et du
670 régime algal. Université de Bretagne Occidentale, Brest.
- 671 Richard, G., Le Bris, C., Guerard, F., Lambert, C., Paillard, C., 2015. Immune responses of
672 phenoloxidase and superoxide dismutase in the manila clam *Venerupis philippinarum*

- 673 challenged with *Vibrio tapetis* - Part II: Combined effect of temperature and two *V.*
674 *tapetis* strains. Fish Shellfish Immunol. 44, 79–87. doi:10.1016/j.fsi.2014.12.039
- 675 Sies, H., 1993. Strategies of Antioxidant Defense. Eur. J. Biochem. 215, 213–219.
676 doi:10.1111/j.1432-1033.1993.tb18025.x
- 677 Smith, A.D., Levander, O.A., 2002. High-throughput 96-well microplate assays for
678 determining specific activities of glutathione peroxidase and thioredoxin reductase.
679 Protein Sens. React. Oxyg. Species Pt Selenoproteins Thioredoxin 347, 113–121.
- 680 Soudant, P., Chu, F.-L.E., Volety, A., 2013. Host-parasite interactions: Marine bivalve molluscs
681 and protozoan parasites, *Perkinsus* species. J. Invertebr. Pathol. 114, 196–216.
682 doi:10.1016/j.jip.2013.06.001
- 683 Soudant, P., Marty, Y., Moal, J., Robert, R., Quere, C., LeCoz, J.R., Samain, J.F., 1996. Effect of
684 food fatty acid and sterol quality on *Pecten maximus* gonad composition and
685 reproduction process. Aquaculture 143, 361–378. doi:10.1016/0044-8486(96)01276-
686 8
- 687 Soudant, P., Van Ryckeghem, K., Marty, Y., Moal, J., Samain, J.F., Sorgeloos, P., 1999.
688 Comparison of the lipid class and fatty acid composition between a reproductive
689 cycle in nature and a standard hatchery conditioning of the Pacific Oyster *Crassostrea*
690 *gigas*. Comp. Biochem. Physiol. B-Biochem. Mol. Biol. 123, 209–222.
691 doi:10.1016/S0305-0491(99)00063-2
- 692 Tafalla, C., Novoa, B., Figueras, A., 2002. Production of nitric oxide by mussel (*Mytilus*
693 *galloprovincialis*) hemocytes and effect of exogenous nitric oxide on phagocytic
694 functions. Comp. Biochem. Physiol. B-Biochem. Mol. Biol. 132, 423–431.
- 695 Torreilles, J., Guerin, M.C., 1999. Production of peroxynitrite by zymosan stimulation of
696 *Mytilus galloprovincialis* haemocytes *in vitro*. Fish Shellfish Immunol. 9, 509–518.
697 doi:10.1006/fsim.1998.0200
- 698 Trinkler, N., Siquin, G., Querne, J., Paillard, C., 2010. Resistance to Brown Ring Disease in
699 the Manila clam, *Ruditapes philippinarum*: A study of selected stocks showing a
700 recovery process by shell repair. J. Invertebr. Pathol. 104, 8–16.
701 doi:10.1016/j.jip.2009.12.007
- 702 Umasuthan, N., Bathige, S.D.N.K., Revathy, K.S., Lee, Y., Whang, I., Choi, C.Y., Park, H.-C., Lee,
703 J., 2012a. A manganese superoxide dismutase (MnSOD) from *Ruditapes*
704 *philippinarum*: Comparative structural- and expressional-analysis with copper/zinc
705 superoxide dismutase (Cu/ZnSOD) and biochemical analysis of its antioxidant
706 activities. Fish Shellfish Immunol. 33, 753–765. doi:10.1016/j.fsi.2012.06.024
- 707 Umasuthan, N., Revathy, K.S., Lee, Y., Whang, I., Choi, C.Y., Lee, J., 2012b. A novel molluscan
708 sigma-like glutathione S-transferase from Manila clam, *Ruditapes philippinarum*:
709 Cloning, characterization and transcriptional profiling. Comp. Biochem. Physiol. C-
710 Toxicol. Pharmacol. 155, 539–550. doi:10.1016/j.cbpc.2012.01.001
- 711 Umasuthan, N., Revathy, K.S., Lee, Y., Whang, I., Lee, J., 2012c. Mitochondrial thioredoxin-2
712 from Manila clam (*Ruditapes philippinarum*) is a potent antioxidant enzyme involved
713 in antibacterial response. Fish Shellfish Immunol. 32, 513–523.
714 doi:10.1016/j.fsi.2011.12.010
- 715 Viarengo, A., Canesi, L., Pertica, M., Livingstone, D., Orunesu, M., 1991. Age-Related Lipid-
716 Peroxidation in the Digestive Gland of Mussels - the Role of the Antioxidant Defense
717 Systems. Experientia 47, 454–457. doi:10.1007/BF01959942

- 718 Villamil, L., Gomez-Leon, J., Gomez-Chiarri, M., 2007. Role of nitric oxide in the defenses of
719 *Crassostrea virginica* to experimental infection with the protozoan parasite *Perkinsus*
720 *marinus*. Dev. Comp. Immunol. 31, 968–977. doi:10.1016/j.dci.2007.01.006
- 721 Wang, C., Huan, P., Yue, X., Yan, M., Liu, B., 2011. Molecular characterization of a glutathione
722 peroxidase gene and its expression in the selected *Vibrio*-resistant population of the
723 clam *Meretrix meretrix*. Fish Shellfish Immunol. 30, 1294–1302.
724 doi:10.1016/j.fsi.2011.03.015
- 725 Wang, C., Yue, X., Lu, X., Liu, B., 2013. The role of catalase in the immune response to
726 oxidative stress and pathogen challenge in the clam *Meretrix meretrix*. Fish Shellfish
727 Immunol. 34, 91–99. doi:10.1016/j.fsi.2012.10.013
- 728 Wickham, H., Chang, W., 2015. ggplot2: An Implementation of the Grammar of Graphics.
- 729 Winston, G., Digiulio, R., 1991. Prooxidant and Antioxidant Mechanisms in Aquatic
730 Organisms. Aquat. Toxicol. 19, 137–161. doi:10.1016/0166-445X(91)90033-6
731
732

733 List of figures

734 Figure 1: Commonly accepted enzymatic antioxidant pathways involved in reactive oxygen
735 species (in red) and reactive nitrogen species (in blue) production and detoxication in bivalve
736 mollusks hemocytes, in intracellular compartment (mitochondria, peroxisome, cytosol) and
737 in extracellular compartment (plasma). Abbreviations: NADPH-Ox, NADPH oxidase; Cu/Zn-
738 SOD, Cu/Zn superoxide dismutase; Mn-SOD, Mn superoxide dismutase; EC-SOD, extracellular
739 superoxide dismutase; CAT, catalase; GPx, glutathion peroxidase; GR, glutathion reductase;
740 GST, glutathione-S-transferase; GSSG, oxidized glutathione; GSH, reduced glutathione; TrxP;
741 thioredoxin peroxidase; TrxR, thioredoxin reductase; Trx(ox), oxidized thioredoxin; Trx(red),
742 reduced thioredoxin; iNOS, inducible nitric oxide synthase; $O_2^{\bullet-}$, superoxide; H_2O_2 , hydrogen
743 peroxide; ROOH, organic peroxides; ROH, alcohols; NO^{\bullet} , nitric oxide; $ONOO^-$, peroxynitrite;
744 NO_2^- , nitrite; L-ARG, L-Arginine; L-CIT, L-Citrulline.

745 Figure 2: Principal component analysis ordination biplot representing the effect of treatment
746 on biochemical parameters of clams (lines) at 2 and 7 days post-injection (dpi). Clams were
747 injected with filtered seawater (control) or with its pathogen *Vibrio tapetis*. Clams injected
748 with *V. tapetis* were either BRD- (no clinical sign of infection) or BRD+ (visible brown ring).
749 White symbols: control; grey symbols: BRD-; black symbols: BRD+. Triangles: 2 dpi; circles: 7
750 dpi. Abbreviations: t-SOD, total superoxide dismutase; Cu/Zn-SOD, Cu/Zn superoxide
751 dismutase; Mn-SOD, Mn superoxide dismutase; CAT, catalase; t-GPx, total glutathion
752 peroxidase; Se-GPx, selenium-dependant glutathione peroxidase; GR, glutathion reductase;
753 GST, glutathione-S-transferase; TrxR, thioredoxin reductase; iNOS, inducible nitric oxide
754 synthase; PO, phenoloxidase; HK, hexokinase ; PK, pyruvate kinase; CS, citrate synthase.

755 Figure 3: Biochemical parameters of clams at 2 and 7 days post-injection (dpi) with filtered
756 seawater (control, white bars) or with *V. tapetis*. Clams injected with *V. tapetis* were either
757 BRD- (no clinical sign of infection, grey bars) or BRD+ (visible brown ring, black bars).
758 Abbreviations: t-SOD, total superoxide dismutase; Cu/Zn SOD; Cu/Zn superoxide dismutase;
759 CAT, catalase; Se-GPx, selenium dependant glutathione peroxidase; GR, glutathione
760 reductase; GST, glutathione-S-transferase; iNOS, inducible nitric oxide synthase; PO,
761 phenoloxidase. Specific enzyme activities are represented as mean \pm standard deviation
762 (SD). Letters indicate significant differences.

763 Appendix A: Inducible Nitric Oxide Synthase activity assay

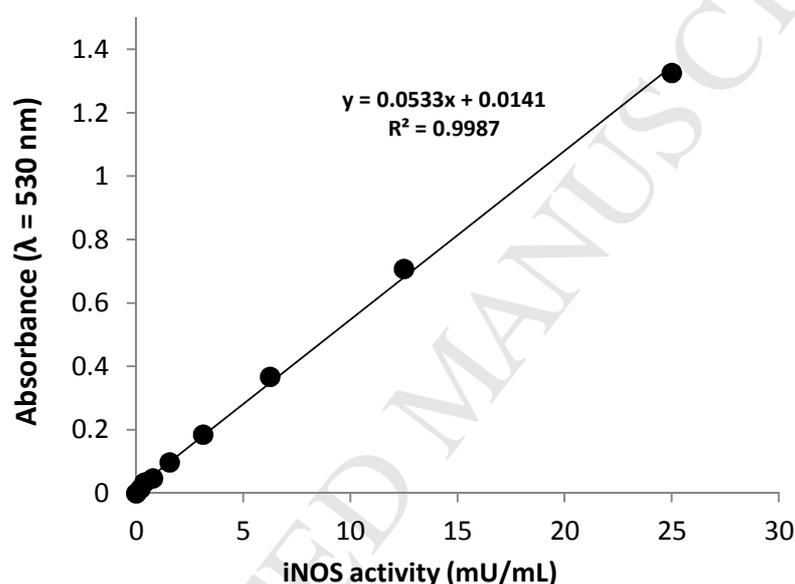
764 Nitric Oxide Synthases (NOS) are NADPH-dependant enzymes that convert L-Arginine
765 (L-ARG) into L-Citrulline (L-CIT) in the presence of its co-factors tetrahydrobiopterin (BH₄) and FAD
766 (Calderon-Cortes et al., 2006). In mammalian systems, three iso-enzymes of NOS are known:
767 the neuronal and constitutive NOS (nNOS or NOS I), the inducible NOS originally found in
768 macrophages (iNOS or NOS II), and the endothelial and constitutive NOS (eNOS or NOS III).
769 Constitutive forms are calmodulin/Ca²⁺ dependant while iNOS is calmodulin/Ca²⁺
770 independent (Knowles and Moncada, 1994). Additionally, iNOS has been characterized in
771 haemocytes of the snail *Viviparus ater* and the oyster *Crassostrea virginica* (Conte and
772 Ottaviani, 1995; Villamil et al., 2007).

773 Numerous studies examining iNOS immune role in bivalves used the Griess Reagent
774 protocol which measures breakdown products of NO (nitrates and nitrites). Thus, this
775 method is not considered as directly evaluating iNOS activity. The most sensitive method for
776 NOS activity determination is based on the monitoring of radiolabelled L-ARG (Knowles and
777 Moncada, 1994). However, this method is time-consuming, due to the chromatographic
778 separation of L-CIT from L-ARG (Knipp and Vasak, 2000).

779 Here, we propose a protocol for determination of iNOS activity which is non-
780 radioactive and directly measures the enzyme's product, L-CIT. Our method is based on that
781 of Knipp and Vasak (Knipp and Vasak, 2000) and relies on L-citrulline formation detection.

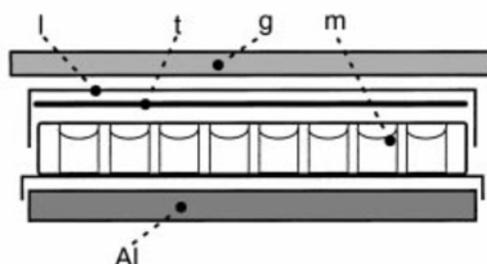
782 Briefly, 55 µL of protein supernatants were incubated during 30 minutes at 37°C with
783 5 µL of assay buffer: Tris buffer (20 mM pH 7.4), NADPH (1 mM), L-Arginine (1 mM), 5,6,7,8-
784 tetrahydrobiopterin (10 µM), FAD⁺ (5 µM). As iNOS is Ca²⁺/Calmodulin independent,
785 contrarily to constitutive NOS, these reagents were not added to the reaction buffer (Cho et
786 al., 1992; Gross, 1996). Samples were also incubated with the same assay buffer containing
787 L-NMMA (200 µM), a specific inhibitor of NOS, in order to assess specifically NOS activity
788 (Knowles and Moncada, 1994). Blanks were run (PBS buffer instead of samples) and iNOS
789 from mouse was used as a standard for range curve (Figure 1). The reactions were stopped
790 by addition of 200 µL of the colour developing reagent (COLDER): 2, 3-butanedione
791 monoxime (20 mM), thiosemicarbazide (0.5 mM), phosphoric acid (2.25 M), sulfuric acid (4.5
792 M), ammonium iron (III) sulfate dodecahydrate (1.5 mM). Then, the microplate was

793 immediately sealed with foil and placed on a preheated aluminium plate in an oven at 95°C
 794 for 15 minutes for colour development (Figure 2). After colour development, the microplate
 795 was removed from the oven and allowed to cool down for 10 minutes at room temperature.
 796 Absorbances were read at 530 nm. Activities were calculated for each sample by
 797 subtracting blank values and activities obtained with L-NMMA inhibition, and expressed as
 798 U/mg total protein (1 U corresponding to the amount of enzyme producing 1 µmole of L-
 799 citrulline per minute in the above conditions).



800

801 Figure 1: Example of standard curve obtained with iNOS from mouse following this protocol.



802 Figure 2: Setup for colour development – from Knipp & Vasak. 2000.

803 Abbreviations: m = microplate; l = lid of the microplate; t = thermoresistant sealing tape (foil
 804 tape); Al = preheated aluminum plate (at 95°C); g = preheated glass plate (at 95°C).

805 **References**

- 806 Calderon-Cortes, E., Clemente-Guerrero, M., Sierra-Campos, E., Cortes-Rojo, C., Gaona-
807 Zamudio, F.J., Villalobos-Molina, R., Saavedra-Molina, A., 2006. Functional
808 characterization of brain mitochondrial nitric oxide synthase during hypertension and
809 aging. *Amino Acids* 30, 73–80. doi:10.1007/s00726-005-0213-x
- 810 Cho, H., Xie, Q., Calaycay, J., Mumford, R., Swiderek, K., Lee, T., Nathan, C., 1992. Calmodulin
811 Is a Subunit of Nitric Oxide Synthase from Macrophages. *J. Exp. Med.* 176, 599–604.
812 doi:10.1084/jem.176.2.599
- 813 Conte, A., Ottaviani, E., 1995. Nitric Oxide Synthase Activity in Molluscan Hemocytes. *Febs*
814 *Lett.* 365, 120–124. doi:10.1016/0014-5793(95)00439-G
- 815 Gross, S.S., 1996. Microtiter plate assay for determining kinetics of nitric oxide synthesis.
816 *Nitric Oxide Pt - Sources Detect. No No Synthase* 268, 159–168.
- 817 Knipp, M., Vasak, M., 2000. A colorimetric 96-well microtiter plate assay for the
818 determination of enzymatically formed citrulline. *Anal. Biochem.* 286, 257–264.
819 doi:10.1006/abio.2000.4805
- 820 Knowles, R., Moncada, S., 1994. Nitric Oxide Synthases in Mammals. *Biochem. J.* 298, 249–
821 258.
- 822 Villamil, L., Gomez-Leon, J., Gomez-Chiarri, M., 2007. Role of nitric oxide in the defenses of
823 *Crassostrea virginica* to experimental infection with the protozoan parasite *Perkinsus*
824 *marinus*. *Dev. Comp. Immunol.* 31, 968–977. doi:10.1016/j.dci.2007.01.006
- 825
- 826

827 **Appendix B: Summaries of means \pm standard deviation and ANOVA for biochemical**
 828 **parameters of clams at 2 and 7 days post-injection (dpi) with filtered seawater (control) or**
 829 **with *V. tapetis*. Clams injected with *V. tapetis* were either BRD- (no clinical sign of infection)**
 830 **or BRD+ (visible brown ring). Abbreviations: t-SOD, total superoxide dismutase; Cu/Zn-SOD,**
 831 **Cu/Zn superoxide dismutase; Mn-SOD, Mn superoxide dismutase; CAT, catalase; t-GPx,**
 832 **total glutathion peroxidase; Se-GPx, selenium-dependant glutathione peroxidase; GR,**
 833 **glutathion reductase; GST, glutathione-S-transferase; TrxR, thioredoxin reductase; iNOS,**
 834 **inducible nitric oxide synthase; PO, phenoloxidase; HK, hexokinase; PK, pyruvate kinase;**
 835 **CS, citrate synthase. Letters indicate significant differences.**

Variable	Unit	2 dpi		7 dpi			ANOVA statistics	
		Control	BRD-	Control	BRD-	BRD+	F	p-value
t-SOD	U/mg	1.49 \pm 0.08 ^{ab}	1.32 \pm 0.11 ^b	1.37 \pm 0.16 ^b	1.76 \pm 0.11 ^a	1.45 \pm 0.19 ^b	4.5	<0.01
Cu/Zn-SOD	U/mg	0.87 \pm 0.34	0.67 \pm 0.21	0.67 \pm 0.24	1.15 \pm 0.09	0.80 \pm 0.26	1.73	0.18
Mn-SOD	U/mg	0.61 \pm 0.31	0.65 \pm 0.22	0.75 \pm 0.30	0.60 \pm 0.02	0.60 \pm 0.21	0.46	0.77
CAT	U/mg	13.25 \pm 2.16 ^b	18.05 \pm 4.52 ^{ab}	15.92 \pm 2.14 ^b	26.82 \pm 13.15 ^a	14.37 \pm 2.00 ^b	5.87	<0.01
t-GPx	mU/mg	9.34 \pm 1.05	8.01 \pm 1.38	9.32 \pm 1.59	6.75 \pm 0.77	8.01 \pm 1.59	2.06	0.13
Se-GPx	mU/mg	2.43 \pm 0.49 ^a	1.32 \pm 0.54 ^b	1.59 \pm 0.70 ^{ab}	2.41 \pm 0.26 ^a	1.11 \pm 0.58 ^b	6.76	<0.01
GR	mU/mg	9.09 \pm 1.74 ^c	10.55 \pm 1.56 ^{bc}	12.18 \pm 1.92 ^b	15.69 \pm 0.06 ^a	11.34 \pm 1.20 ^{bc}	8.32	<0.01
GST	mU/mg	74.36 \pm 8.47	92.64 \pm 25.99	101.32 \pm 23.81	112.00 \pm 29.85	72.03 \pm 21.88	3.19	0.03
TrxR	mU/mg	269.23 \pm 72.51	181.62 \pm 48.19	215.80 \pm 100.14	125.76 \pm 47.75	228.50 \pm 91.13	1.96	0.13
iNOS	mU/mg	5.88 \pm 0.84 ^c	7.76 \pm 0.91 ^{ab}	7.32 \pm 1.32 ^{bc}	6.79 \pm 0.75 ^{bc}	9.20 \pm 1.41 ^a	8.43	<0.01
PO	I.U.	0.10 \pm 0.03 ^b	0.09 \pm 0.02 ^b	0.10 \pm 0.02 ^b	0.15 \pm 0.01 ^a	0.09 \pm 0.02 ^b	3.23	0.03
HK	U/mg	6.05 \pm 1.34	6.00 \pm 0.68	6.77 \pm 0.71	6.91 \pm 2.16	7.29 \pm 0.97	1.99	0.13
PK	mU/mg	26.33 \pm 3.69	22.93 \pm 3.16	26.41 \pm 1.52	27.62 \pm 3.32	26.75 \pm 2.76	2.18	0.10
CS	mU/mg	31.92 \pm 7.27	43.92 \pm 21.90	54.24 \pm 15.05	47.53 \pm 1.19	58.47 \pm 12.41	2.21	0.16
Proteins	mg/g WW	21.80 \pm 0.81 ^{ab}	21.13 \pm 1.43 ^{ab}	21.0 \pm 0.41 ^{ab}	16.71 \pm 3.27 ^b	23.91 \pm 0.72 ^a	3.57	0.02
Carbohydrates	mg/g WW	13.71 \pm 5.04	17.48 \pm 5.13	15.40 \pm 4.85	11.01 \pm 5.21	18.32 \pm 4.74	1.49	0.24

836

Table 1: Sampling design. Clams were fed *T-Isochrysis aff. galbana*, clone Tahitian (*T-Iso*) or *Chaetoceros calcitrans* (*C. calcitrans*) and injected with *Vibrio tapetis* or filtered sea-water (FSW, Control). Clams injected with *V. tapetis* were either BRD- (no clinical sign of infection) or BRD+ (visible brown ring). Abbreviations: P, pool number; n, number of individuals for the pool; T, tank number; dpi, days post-injection.

Diet	Injection	BRD status	Time (dpi)		
			2	7	30
<i>C. calcitrans</i>	FSW	Control	P1 n=3 T4	P1 n=2 T4	P1 n=2 T4
			P2 n=3 T4	P2 n=2 T5	P2 n=3 T5
			P3 n=3 T5	P3 n=3 T5	P3 n=4 T6
			P4 n=3 T6	P4 n=3 T6	P4 n=3 T6
<i>C. calcitrans</i>	<i>V. tapetis</i>	BRD-	P1 n=3 T1		
			P2 n=3 T2		
			P3 n=3 T3		
<i>C. calcitrans</i>	<i>V. tapetis</i>	BRD+		P1 n=3 T1	
				P2 n=3 T2	P1 n=3 T1
				P3 n=4 T2	P2 n=3 T2
				P4 n=3 T3	P3 n=4 T3
				P5 n=3 T3	
<i>T-Iso</i>	FSW	Control	P1 n=3 T13	P1 n=2 T13	
			P2 n=3 T14	P2 n=3 T14	P1 n=3 T14
			P3 n=3 T14	P3 n=4 T15	P2 n=3 T15
			P4 n=3 T15		
<i>T-Iso</i>	<i>V. tapetis</i>	BRD-	P1 n=3 T10		
			P2 n=3 T10	P1 n=2 T10	
			P3 n=5 T11	P2 n=2 T10	
			P4 n=3 T12		
<i>T-Iso</i>	<i>V. tapetis</i>	BRD+		P1 n=3 T11	P1 n=5 T10
				P2 n=3 T12	P2 n=4 T11

Table 2: Percent composition of the main long-chain polyunsaturated fatty acids in total lipids of *T-Isochrysis aff. galbana*, clone Tahitian (*T-Iso*) and *Chaetoceros calcitrans* (*C. calcitrans*) and neutral lipids of the digestive gland of control clams (DG) at 2 dpi. Values are mean \pm standard deviations, expressed as percentage of total fatty acids (n=3 samples).

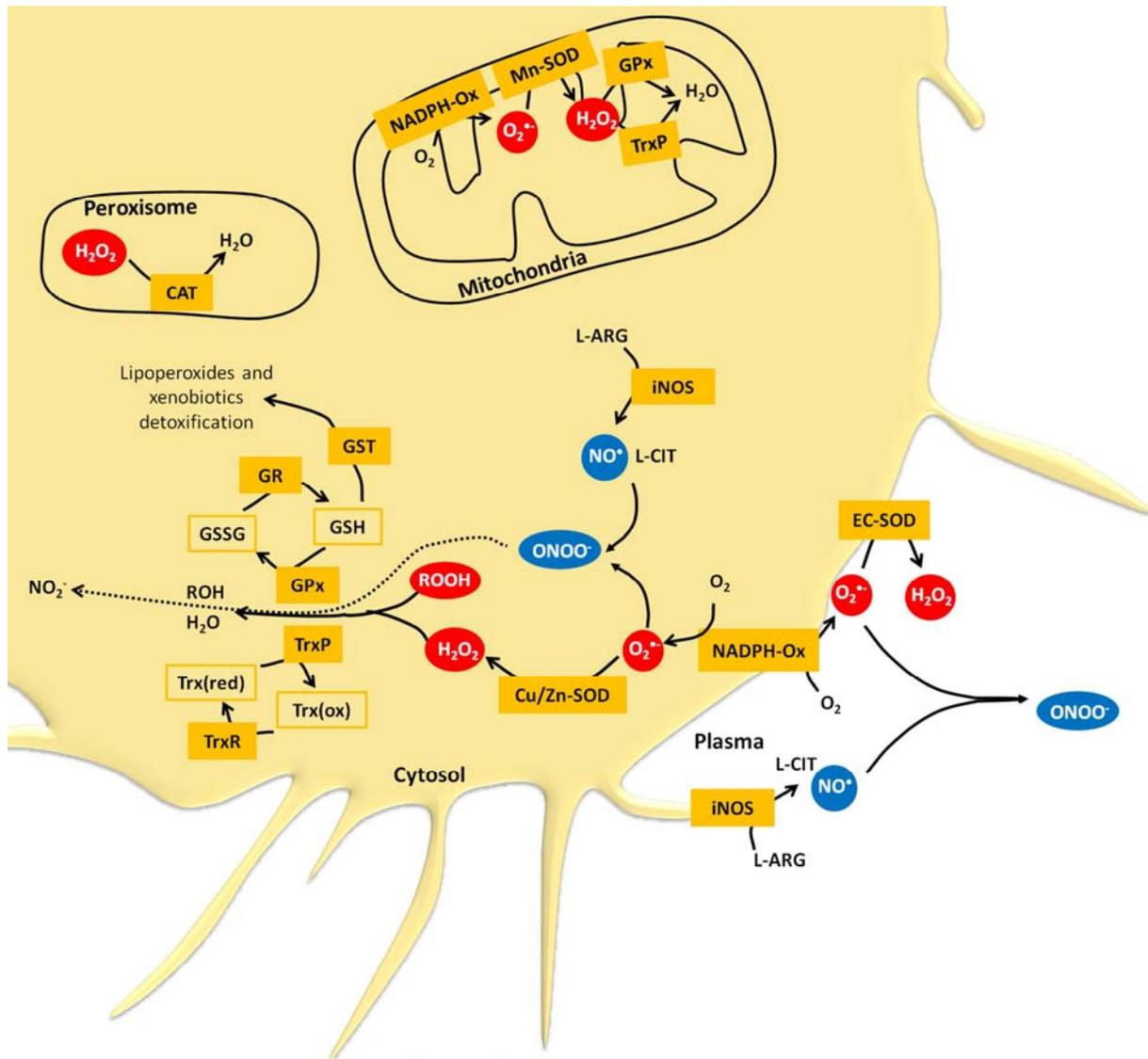
Fatty acid	Algae		DG	
	<i>C. calcitrans</i>	<i>T-Iso</i>	<i>C. calcitrans</i>	<i>T-Iso</i>
20:4n-6	9.2 \pm 3.3	3.1 \pm 0.7	4.6 \pm 0.6	1.2 \pm 0.2
20:5n-3	14.0 \pm 1.4	0.4 \pm 0.1	12.6 \pm 2.1	2.0 \pm 1.3
22:6n-3	1.3 \pm 0.1	8.4 \pm 0.4	5.1 \pm 1.0	14.1 \pm 1.4

Table 3: Percent composition of the main long-chain polyunsaturated fatty acids in polar lipids of clam mantle collected at 7 dpi. Clams were fed *Chaetoceros calcitrans* or T-*Isochrysis aff. galbana*, clone Tahitian (T-*Iso*) and injected with filtered seawater (control) or *Vibrio tapetis*. Clams injected with *V. tapetis* were either BRD- (no clinical sign of infection) or BRD+ (visible brown ring). Values are mean \pm standard deviations, expressed as percentage of total fatty acids.

Fatty acid	<i>C. calcitrans</i>		T- <i>Iso</i>		
	Control	BRD+	Control	BRD-	BRD+
20:4n-6	5.4 \pm 0.5	5.4 \pm 0.4	5.0 \pm 0.4	5.0 \pm 0.8	4.9 \pm 0.2
20:5n-3	7.4 \pm 0.5	7.2 \pm 0.4	5.7 \pm 0.3	5.3 \pm 0.5	5.8 \pm 0.1
22:6n-3	19.5 \pm 0.8	19.8 \pm 0.6	21.6 \pm 1.5	20.6 \pm 1.0	21.0 \pm 0.7
Unsaturation index	277 \pm 3.4	274 \pm 4.6	282 \pm 8.6	277 \pm 0.7	282 \pm 4.2

Table 4: Summary of PERMANOVA conducted on different sampling times (significant effects are in bold).

Times	Explanatory variables	PERMANOVA statistics		
		r ²	F	p-value
2, 7 and 30 dpi	Diet	0.01	0.54	0.64
	Treatment	0.30	2.19	0.03
	Diet x Treatment	0.20	1.78	0.09
2 and 7 dpi	Diet	0.04	1.7	0.16
	Treatment	0.36	3.89	<0.01
	Diet x Treatment	0.28	4.08	<0.01
2 dpi	Diet	0.03	0.65	0.53
	Injection x BRD status	0.29	5.8	0.01
	Diet x Injection x BRD status	0.14	2.86	0.07
7 dpi	Diet	0.07	1.24	0.32
	Injection x BRD status	0.3	2.68	0.03
	Diet x Injection x BRD status	0.02	0.36	0.81
30 dpi	Diet	0.06	0.51	0.66
	Injection x BRD status	0.03	0.23	0.92
	Diet x Injection x BRD status	0.06	0.52	0.63



ACCEPTED

