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## Cellular and molecular complementary immune stress markers for the model species *Dreissena polymorpha*

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1 Full title: **Cellular and molecular complementary immune stress markers for the model**  
2 **species *Dreissena polymorpha*.**

3

4 Short title: **New insight on hemocyte markers in zebra mussel.**

5

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27 **Abstract**

28 This study aimed to combine cellular and molecular analyses for better detail the effects of  
29 various stresses on a sentinel species of freshwater invertebrate. For this purpose, the  
30 hemocytes of the zebra mussel, *Dreissena polymorpha*, were exposed to different stresses  
31 at two different intensities, high or low: chemical (cadmium and ionomycin), physical  
32 (ultraviolet B), or biological ones (*Cryptosporidium parvum* and *Toxoplasma gondii*). After  
33 exposure, flow cytometry and droplet digital PCR analyses were performed on the same  
34 pools of hemocytes. Several responses related to necrosis, apoptosis, phagocytosis,  
35 production of nitric oxide and expression level of several genes related to the antioxidant,  
36 detoxification and immune systems were evaluated. Results showed that hemocyte integrity  
37 was compromised by both chemical and physical stress, and cellular markers of  
38 phagocytosis reacted to ionomycin and protozoa. While cadmium induced oxidative stress  
39 and necrosis, ionomycin tends to modulate the immune response of hemocytes. Although  
40 both biological stresses led to a similar immune response, *C. parvum* oocysts induced more  
41 effects than *T. gondii*, notably through the expression of effector caspases gene and an  
42 increase in hemocyte necrosis. This suggests different management of the two protozoa by  
43 the cell. This work provides new knowledge of biomarkers in the zebra mussel, at both  
44 cellular and molecular levels, and contributes to elucidate the mechanisms of action of  
45 different kinds of stress in this species.

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47 **Keywords:** Bivalve; ddPCR; flow cytometry; hemocyte; protozoa.

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## 55 **1. Introduction**

56 Over the last years, ecotoxicology has gained increasing interest to assess the impact of  
57 anthropogenic contaminants on organisms and ecosystems. Among the tools developed for  
58 environmental risk assessment, biomarkers are of great interest, especially for their potential  
59 as early warning indicators of contamination [1]. These tools are therefore increasingly  
60 developed on sentinel organisms to better characterize the potential effects of contaminants.  
61 In conjunction with chemical and biocenotic analyses, biomarkers contribute to being aware  
62 of the global impact of this contamination on ecosystems.

63 However, this risk prediction requires a good knowledge of the biomarkers used as well as  
64 an explanation of the mechanical bases of xenobiotic effects on well-known sentinel species  
65 [2]. It is therefore essential to understand the modes of action of the various xenobiotics at  
66 infra-individual levels (cellular, molecular) before being able to extrapolate to the scale of the  
67 population or the community.

68 Among biomarkers, those linked to the immune system can reflect the state of health of an  
69 organism under stress. Indeed, the immune system is responsible for the integrity of  
70 organism, protects it against infections and parasitism and is linked to various other  
71 biological functions, such as nervous or endocrine systems [3-6]. These immune markers are  
72 often i) functional and assessed at the cellular level, such as the capacity or the efficiency of  
73 phagocytosis, ii) structural in terms of the integrity of the immune effector cells, or iii) linked to  
74 the expression of genes coding for immune proteins. They are therefore mainly assessed on  
75 two levels of biological organization: the cell and the molecule. The analysis of multi-level  
76 markers has several advantages. First, it allows a more complete diagnosis of contamination  
77 effects on an organism and can allow their extrapolation on a population [7, 8]. Then, this  
78 multi-level analysis can provide information on the impact kinetics of a contamination within  
79 an organism [9]. Finally, it can be used to determine whether the responses are due to the  
80 exposure or only due to a homeostatic modification [10].

81 In this study, cellular and molecular analyses were combined to understand the effects of  
82 various stress on a sentinel species of freshwater invertebrate, the zebra mussel *Dreissena*  
83 *polymorpha*. This invasive species is known to have colonized several regions in the  
84 northern hemisphere (America, Europe) and to have negatively impacted the invaded  
85 ecosystems [11]. Our objective was to understand the immune response capabilities of  
86 hemocytes of the zebra mussel to various stresses (chemical, physical and biological), *via*  
87 cellular and molecular markers. For this purpose, several parameters were set upstream of  
88 the study. First, bivalves were chosen because they have several advantages to be used in  
89 biomonitoring programs as sentinel species [12-15]: i) these animals are directly in contact  
90 with the contamination (water and sediment) and can accumulate pollutants [16]; ii) they are  
91 sessile, making possible a spatial and temporal assessment of surrounding contamination  
92 [13, 14, 17]. For freshwater biomonitoring, *Dreissena polymorpha* is a good sentinel species  
93 since it occupies a large geographical area, it resists to variations in external conditions and  
94 is considered to be the counterpart of *Mytilus edulis* for ecotoxicological studies [18-20].  
95 Second, cellular and molecular markers were evaluated on the same measurement scale,  
96 namely on hemocytes. In bivalves, hemocytes are the effector cells of immunity. Exposures  
97 to various stresses were conducted in *ex vivo* conditions to overcome the complexity of the  
98 whole organism and to minimize the exposure bias. Third, all analyses (cellular and  
99 molecular ones) were performed on the same samples to provide a better comprehension of  
100 stressors' modes of action. This criterion required to work with pools of hemolymph to obtain  
101 enough biological material for analysis. Finally, hemocytes from *D. polymorpha* were  
102 exposed to various stresses (chemical, physical and biological), whose expected effects are  
103 known. Hemocytes were exposed to i) cadmium, a cytotoxic metal [21]; ii) ionomycin, an  
104 antibiotic which induces apoptosis and alters immune functions [22, 23]; iii) ultra-violet B  
105 (UVb) which are known to be cytotoxic and immune suppressor [24, 25]; and iv) two  
106 protozoa, *Cryptosporidium parvum* and *Toxoplasma gondii*, whose effects on the hemocytes  
107 of mussels have already been evaluated [26].

108 Regarding mussel immunity, two processes have been studied *via* cellular and molecular  
109 markers, namely:

110 - Phagocytosis, through the internalization of microbeads (capacity, efficiency and  
111 avidity of phagocytosis) and the indirect stimulating action of nitric oxide (NO, [27]) for  
112 cellular markers, as well as by the expression of several genes involved in this response: the  
113 complex *arp 2/3*, involved in the regulation of cytoskeleton actin; *cdc 42*, involved in cell  
114 migration and endocytosis; and *gal 2*, a galactose-binding lectin, playing many roles  
115 including cell adhesion and signalling [28-30].

116 - Apoptosis, *via* the study of the cellular activation of an effector with cysteine effector,  
117 caspases 3/7, and the expression of its gene. Galectin-2 also plays a role in apoptosis by  
118 being part of anti-apoptotic molecules [31].

119 The molecular markers were selected in accordance with the work of Leprêtre *et al.* [32] who  
120 observed the involvement of the proteins encoded by these genes in the cell compartment of  
121 the hemolymph of the zebra mussel.

122 Cell necrosis marker, detoxification and antioxidant systems were also evaluated, by flow  
123 cytometry and droplet digital polymerase chain reaction (ddPCR). This latter technology is  
124 based on the massive fragmentation of the genetic material, which facilitates the analysis of  
125 low-expressed genes [33, 34]. ddPCR was preferred to other PCR techniques because of  
126 the low expression levels of certain genes such as *cdc 42* or *casp 3/7*, as well as being able  
127 to express the absolute result in a number of copies per hemocyte. Thanks to the partitioning  
128 of the sample and Poisson's law, ddPCR makes possible to express the quantification results  
129 directly as the number of copies of the target DNA per  $\mu\text{L}$ , or in our case as the number of  
130 copies per cell. This technique improves the measurement accuracy without the need for a  
131 prior calibration curve [34, 35]. This simplifies quantification and reduces the risk of error.  
132 Compared to other PCR techniques, it offers other advantages such as high accuracy and an  
133 increase in the signal-to-noise ratio [34, 36]. This strategy, developed and used in medicine,  
134 is pioneer in ecotoxicology. This opportunity to assess cellular and molecular markers on the

135 same scale, namely the hemocyte, allows a better understanding of the response capacities  
136 of these cells to stress.

137

## 138 **2. Materials and methods**

### 139 *2.1. Mussel and hemolymph collection*

140 Zebra mussels ( $3.18 \pm 0.41$  g;  $2.72 \pm 0.16$  cm) were collected on 25<sup>th</sup> August 2017 from Der  
141 lake (51290 Giffaumont-Champaubert, France, N 48°33'35"; E 4°45'11") and brought back to  
142 the laboratory, where they were maintained in 20 L aerated glass tanks in Cristaline Aurele  
143 drinking water ( $14.41 \pm 0.67$  °C; pH  $7.47 \pm 0.07$ ;  $570 \pm 40$  mS/cm; spring Jandun, France).  
144 Four weeks of acclimation were respected before *ex vivo* experiments. Throughout the study,  
145 mussels were fed, twice per week, with two microalga species: *Scenedesmus obliquus* and  
146 *Chlorella pyrenoidosa* ( $10^6$  microalgae of each species per mussel and per day). Water (6 L)  
147 was changed every week, after assessment of nitrites ( $0.12 \pm 0.02$  mg/L), nitrates ( $34.32 \pm$   
148  $13.16$  mg/L), and ammonium concentrations ( $0.03 \pm 0.01$  mg/L).

149 Hemolymph recovery was realised before feeding to not compromise cytometric analyses.  
150 Hemolymph from ten mussels was withdrawn from adductor muscle with 0.3 mL syringes (30  
151 G x 8 mm, Becton Dickinson, NJ, USA), kept on ice and pooled to obtain enough hemolymph  
152 volume for both flow cytometric and ddPCR analysis. Cell concentration was determined with  
153 KOVA slide (KOVA International, CA, USA), then adjusted to 375,000 cells per mL and  
154 samples were separated into microplate (U-bottom, VWR, PA, USA) for flow cytometry and  
155 into tubes for ddPCR. To discard cell debris and dead cells caused by sampling and starting  
156 with healthy samples, plasma was removed after 20 minutes of hemocyte sedimentation (16  
157 °C, in the dark [37]). Then, 190 µL (or 200 µL for UVb exposure) of modified Leibovitz 15  
158 medium (L15, Sigma-Aldrich, MO, USA) were added for flow cytometry [38, 39]. For ddPCR  
159 analyses, samples were placed into three 1.5 mL tubes with phosphate buffer saline (Sigma-  
160 Aldrich) q.s. 500 µL per tube.

161

162 *2.2. Nature of stress and exposure*

163 Stresses of different natures were selected: two chemical ones, represented by cadmium  
 164 chloride (Agilent technologies, CA, USA) and an antibiotic from *Streptomyces globatus*  
 165 named ionomycin (VWR); a physical stress with exposure to ultraviolet B (UVb, 254 nm, at a  
 166 distance of 10 cm, tubes or plates closed to prevent evaporation and kept on ice, Camag,  
 167 NC, USA). Finally, two human protozoan parasites were selected as biological stressors,  
 168 *Toxoplasma gondii* (generously gifted by J.P. Dubey, USDA, Beltsville, USA) and  
 169 *Cryptosporidium parvum* (INRAE, Val de Loire research center, France). Hemocytes were  
 170 submitted to the different stress conditions for four hours (Table 1): i) to each solvent for  
 171 control conditions, ii) to cadmium chloride (in phosphate buffer saline, Sigma-Aldrich), to  
 172 ionomycin (in phosphate buffer saline with 0.2 % dimethylsulfoxide, ThermoFisher Scientific,  
 173 MA, USA) or to protozoan solutions (in phosphate buffer saline), and iii) hemocytes were  
 174 exposed to a UVb light for 0 (control), 3 or 10 minutes.

175

176 **Table 1:** Nature and intensities of each stress imposed on zebra mussel hemocytes.

177

Stress nature	Stress intensity			Reference
	null	medium	high	
Cadmium chloride µg/L	0	25	250	Sokolova <i>et al.</i> (2004) Fang <i>et al.</i> (2010)
Ionomycin µM	0	1	10	Opper <i>et al.</i> (2010)
Ultraviolet B min	0	3	10	Estevez-Calvar <i>et al.</i> (2013)
<i>Toxoplasma gondii</i> protozoa:cell ratio	0:1	1:25	1:5	Le Guernic <i>et al.</i> (2020)
<i>Cryptosporidium parvum</i> protozoa:cell ratio	0:1	1:1	5:1	Le Guernic <i>et al.</i> (2020)

178

179 *2.3. Flow cytometric analyses*

180 Analyses with flow cytometer were based on Barjhoux *et al.* [37] protocol with minor  
 181 modifications. Briefly, hemocyte samples were placed in nine wells (three stress conditions x

182 three analyses), at 75.000 cells per well. After UVb exposure or immediately after adding  
 183 other stressors, hemocytes were incubated four hours according to the following conditions:  
 184 i) 2 µm diameter yellow-green fluorescent latex microbeads with a cell:bead ratio of 1:50 (16  
 185 °C, in the dark, Polysciences Inc., PA, USA, [37]), ii) 0.5 µM caspases 3/7 green flow  
 186 cytometric reagent (30 min, 16 °C, in the dark, Thermofisher Scientific), and iii) with 5 µM of  
 187 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-fm, 30 min, 16 °C, in the  
 188 dark, Thermofisher Scientific). Ten minutes before analyses, cells were removed into new  
 189 wells with an anti-aggregate solution (Trypsin-EDTA solution, 0.0625 % final, Sigma-Aldrich  
 190 [37]). Just before flow cytometric analyses, propidium iodide (PI, 1 % final, Sigma-Aldrich)  
 191 was added to mark dead cells in double labeling with microbeads. The oocysts were  
 192 removed from flow cytometry analyses by their size, complexity and autofluorescence,  
 193 different from those of hemocytes, as previously describes [26]. Flow cytometric data were  
 194 evaluated with 10,000 cells counted for each suspension sample thanks to Accuri™ C6  
 195 SORP flow cytometer (Becton Dickinson, N.J., USA, with BD Accuri™ C6 software  
 196 (v1.0.264.21)).

197 All immune markers and their corresponding method of analysis are described in Table 2.

198

199 **Table 2:** List of immune markers used in this study with corresponding method of analysis by flow

Immune markers	Abbreviations	Analysis methods	Units	Ex/Em (nm)
Hemocyte necrosis	PI+	percentage of cells labelled with propidium iodide among total cells	%	533/617
Phagocytic capacity	P.cap	percentage of cells that ingested at least one microbead among total cells	%	441/486
Phagocytic efficiency	P.eff	percentage of cells that ingested at least three microbead among total cells	%	441/486
Phagocytic avidity	P.nb	mean number of ingested beads by hemocytes that ingested at least one bead	number	441/486
Hemocyte apoptosis	Casp+	percentage of hemocytes with activated caspases 3/7, fluorescence is induced by cleavage of the associated DEVD peptide by activated caspases.	%	503/530
NO basal production	NO+	DAF-FM is modified to benzotriazole derivative (fluorescent) by the presence of nitric oxide in hemocytes	mean of fluorescence	495/515

200 cytometry, units, excitation wavelength (Ex), and emission wavelength (Em) used.

201

## 202 *2.4. Gene expression analysis*

### 203 2.4.1. RNA extraction and quantification

204 After exposure, the supernatant was removed from the sample after centrifugation (3,000 g,  
205 5 min, 4 °C), and hemocytes were lysed with trizol reagent (Molecular Research Center Inc.,  
206 OH, USA). At this stage, samples were frozen (-80 °C) for several weeks until resumption of  
207 RNA extraction.

208 After thawing, chloroform (VWR) was added and vortexed with samples and then incubated  
209 15 minutes at room temperature. The following steps have been carried out on ice to better  
210 preserve the integrity of the biological material. The aqueous phase containing the nucleic  
211 material was recovered after centrifugation (12,000 g, 8 min, 4 °C). Total RNA was  
212 precipitated using isopropanol (10 minutes on ice, VWR) and the supernatant was kept after  
213 centrifugation (12,000 g, 8 min, 4 °C). RNA was washed with 70 % ethanol (Charbonneaux-  
214 Brabant Chimie, Reims, France), removed from the sample by centrifugation (7,500 g, 5 min,  
215 4 °C). To remove ethanol, the pellet was air-dried on ice for about thirty minutes and was  
216 then mixed with RNase free water (40 µL, United States Biological, MA, USA). A water bath  
217 step was then respected (15 min, 57 °C) to dissolve RNA in water. RNA quantification was  
218 assessed using Genova Nano spectrophotometer (Jenway, Staffordshire, United Kingdom),  
219 and samples were homogenized at 100 ng RNA / µL. Quality index (absorbance ratio  
220  $OD_{260}/OD_{280}$ ) was checked and samples that did not meet quality standards were removed  
221 from the analyses. RNA purity was observed after electrophoresis and UV revelation (1.5 %  
222 agarose gel and TBE buffer (0.5 X, Euromedex, Souffelweyersheim, France)). RNA samples  
223 were frozen (-20 °C) until reverse transcription polymerase chain reaction.

224

### 225 2.4.2. Reverse transcription polymerase chain reaction

226 The reverse transcription was performed with 400 ng of total RNA using Verso cDNA  
 227 Synthesis Kit (ThermoFisher Scientific). The reaction was conducted at 42 °C for 30 min  
 228 followed by 2 minutes at 95 °C (Eppendorf Mastercycler EP Gradient S, Eppendorf France,  
 229 Montesson, France). Reverse transcription polymerase chain reaction was not coupled with  
 230 PCR to reduce final variability [35].

231

### 232 2.4.3. Droplet digital polymerase chain reaction analyses

233 All primers were obtained from Eurogentec (Liège, Belgium). All characteristics concerning  
 234 the primers are present in table 3.

235

236 **Table 3:** List and characteristics of primers used for ddPCR analyses. SOD: superoxide dismutase;  
 237 CAT: catalase; GPx: glutathione peroxidase; GST: glutathione-S-transferase; Casp3/7: caspases 3/7;  
 238 Arp 2/3: actin-related protein 2/3; Cdc 42: cell division control 42; Gal 2: galectin 2. Primers were  
 239 defined via the zebra mussel transcriptome compared to the sequences of the genes of interest in  
 240 other bivalves of the databases.

Primers	Sequences (5'-3')	Amplicon size	Accession reference	Reference
SOD - F100 SOD - R100	GACAGCATGGCTTCCATGTG AGATTCTGGGCCAGTCAGAG	212 pb	AY377970.2	Geret <i>et al.</i> (2004)
CAT - F100 CAT - R100	ACGGCTATGGAAGCCACACG AGGTCGCGCATCGCATAGTC	157 pb	EF681763	Peuthert (2007)
Se - GPx - F100 Se - GPx - R100	TAGACCGCCGCTCGGTAATC CACTCCCAGCACCTGGAAAC	205 pb	EF194204.2	Doyen <i>et al.</i> (2008)
GST - pi -F100 GST - pi -R100	TCCGCTATATCTGCCTGGAC GCTCCTCAGACCTGCTTTC	186 pb	DQ459993	Doyen <i>et al.</i> (2008)
MT - F200 MT - R200	GCGTTGAAACCGGTGATTGC GCCACAACAGTTGGGTTTGC	104 pb	U67347.1	Engelken and Hildebrandt (1999)
Casp3/7 - F Casp3/7 - R	CTCCGTCTAACATACAGGCC GCGTTGACAGAATTCAGTTGC	249 pb		Present study
Arp 2/3 - F Apr 2/3 - R	GCCAAAAGAATTAAGTACA GGGTATTCGGATGTGTGACA	241 pb		Present study
Cdc 42 - F Cdc 42 - R	GCCTAGTGTGGGCACTACAG ACCCACCACTGTCAACGGAC	247 pb		Present study
Gal 2 - F Gal 2 - R	GGAGTCATCATCCACCGTGA CAACGCGGGGCGGAAGCAGA	158 pb		Present study

241

242

243 cDNA samples, diluted to the tenth, were combined according to the manufacturer's  
244 instructions on ice with a mix of primers (final concentration of 150 nM), Eva Green solution  
245 (QX200 ddPCR EvaGreen Supermix RXN, BioRad, CA, USA) and osmosed water. Droplet  
246 formation was then automatized with BioRad apparatus. PCR was then performed within 40  
247 cycles (BioRad thermocycler). All reads with less than 13,000 accepted droplets were  
248 removed from results [35]. The results are then expressed in gene copies per hemocyte, with  
249 reference to the count carried out on each pool.

250 According to the Lievens *et al.* [35] study, many criteria for validating ddPCR data have been  
251 taken into account and are presented in annex 1 (the number of accepted partition; the  
252 number of outliers; the results of NTC, the mean number of copies per partition; the  
253 percentage of "rain" and the maximum threshold).

254

## 255 *2.5. Statistical analysis*

256 Statistical analyses were performed using R 3.4.1 software. An analysis of variance  
257 (ANOVA) was performed and normality and variance homogeneity were tested on its  
258 residuals thanks to Shapiro-Wilk and Levene tests respectively ( $\alpha \leq 5\%$ ). If normality and  
259 homoscedasticity were respected, results from ANOVA were kept and followed by a post-hoc  
260 test to discriminate significant differences inside each group (TukeyHSD with Holm  
261 correction,  $\alpha \leq 5\%$ ). If one of these criteria was not respected, ANOVA was replaced by a  
262 Kruskal-Wallis test followed by Nemenyi post-hoc test ( $\alpha \leq 5\%$ ). Statistics were performed  
263 on ten biological replicas (pool).

264

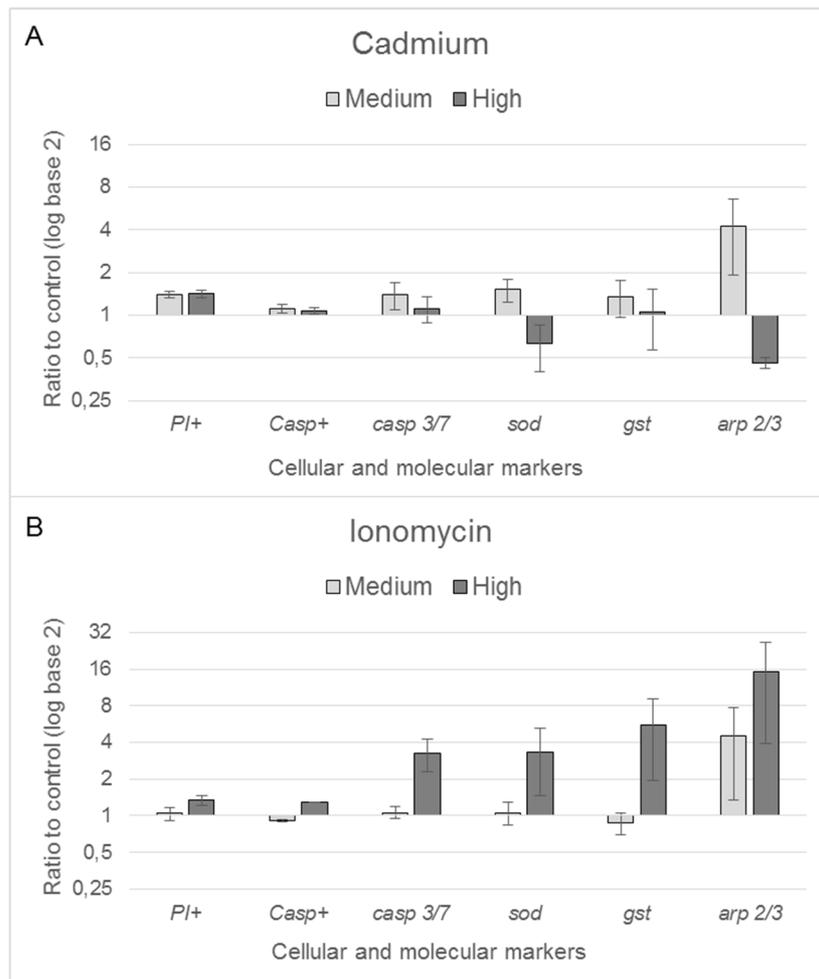
## 265 **3. Results**

### 266 *3.1. Chemical stress (cadmium and ionomycin)*

267 Both chemical stresses were cytotoxic. While cadmium induced hemocyte necrosis from  
268 medium intensity, only the highest concentration of ionomycin was cytotoxic (Table 4).  
269 Contrary to cadmium exposure, ionomycin induced other effects. First, a significant increase

270 of 3/7 caspases activation was observed when the hemocytes were exposed to 10  $\mu$ M  
271 ionomycin compared to the control condition (evolution rate on means: +118.0 %, *p-value* =  
272  $8.40 \times 10^{-3}$ , Table 4). Then, at this concentration, ionomycin also induced a decrease in all  
273 cell markers linked to phagocytosis (the capacity (-23.7 %), efficiency (-56.6 %) and avidity (-  
274 51.7 %) of this immune process, *p-value* =  $4.30 \times 10^{-2}$ ; *p-value* =  $1.00 \times 10^{-2}$ ; *p-value* =  $1.00 \times$   
275  $10^{-2}$ , respectively, Table 4). The intermediate concentration of cadmium chloride induced a  
276 higher *sod* gene expression compared to the control condition (evolution rate: -62.8 %, *p-*  
277 *value* =  $1.67 \times 10^{-2}$ , Table 5) while no significant change was observed for the highest  
278 concentration. No significant effect on gene expression was observed after ionomycin  
279 exposure. Nonetheless, both chemical stressors tended to modify some gene expressions  
280 (Figure 1). At the highest concentrations, opposite effects on gene expression were observed  
281 between cadmium and ionomycin exposures compared to their control. While cadmium  
282 would tend to decrease the gene expression of *sod* (-49.8 %), *gst* (-34.0 %), and *arp 2/3* (-  
283 56.5 %), ionomycin would increase it (*sod* (+163.7 %), *gst* (+152.3 %), *casp 3/7* (+141.2 %)  
284 and *arp 2/3* (+1075.7 %)). This is also observable when the ratios of the two stress intensities  
285 is carried out with respect to the control condition for each pool (Figure 1).

286



287

288 **Figure 1:** Ratio to control (log base 2) for medium and high stresses intensities for each pool (mean ±  
 289 SEM) submitted: A to cadmium and B to ionomycin. The light gray bars correspond to the ratio of  
 290 medium intensity, and the dark gray ones correspond to the ratios of high intensity compared to the  
 291 control. *PI+*: percentage of cells labelled with propidium iodide; *Casp+*: percentage of cells labelled  
 292 with caspases 3/7 reagent; *casp 3/7*: capases 3/7; *sod*: superoxide dismutase; *gst*: glutathione-S-  
 293 transferase; *arp 2/3*: actin-related protein 2/3.

294

295 **3.2. Physical stress (UVb)**

296 From the intermediate intensity (3 min), ultraviolet B induced significant necrosis and  
 297 apoptosis (*via* the activation of 3/7 caspases, Table 4). These effects persisted at the highest  
 298 intensity (10 min). The phagocytic avidity can be impaired by UVb exposure, after 3 (-26.9 %)

299 and 10 minutes (-34.3 %) exposure ( $p$ -value =  $3.04 \times 10^{-2}$  and  $p$ -value =  $8.36 \times 10^{-3}$   
300 compared to control, respectively, Table 4).

301

### 302 3.3. Biological stress (*C. parvum* and *T. gondii*)

303 Contrary to *C. parvum*, oocysts of *T. gondii* did not induce hemocyte necrosis (Table 4).

304 These two protozoa, at the highest stress intensity, disrupted phagocytic parameters: i)

305 phagocytic capacity (evolution rate: -20.3 %,  $p$ -value =  $2.26 \times 10^{-3}$  for *T. gondii* and -27.5 %,  $p$ -value =  $1.20 \times 10^{-2}$  for *C. parvum* compared to control), ii) efficiency (-41.0 %,  $p$ -value =

306  $2.10 \times 10^{-2}$  for *C. parvum* compared to control), and iii) avidity (-24.2 %,  $p$ -value =  $7.78 \times 10^{-3}$

307 for *T. gondii* and -29.0 %,  $p$ -value =  $3.47 \times 10^{-2}$  for *C. parvum* compared to control, Table 4).

308 Finally, exposure to both biological stress at the highest stress intensity induced higher NO

309 basal production than without stress (evolution rate: +126.8 %,  $p$ -value =  $2.50 \times 10^{-3}$  and

310 +41.5 %,  $p$ -value =  $1.24 \times 10^{-2}$ , for *T. gondii* and *C. parvum* respectively, Table 4).

311 In addition to cellular effects, exposure to *C. parvum* oocysts, at the highest stress intensity,

312 induced increase of caspases 3/7 gene expression (+282.3 %,  $p$ -value =  $3.50 \times 10^{-2}$

313 compared to control, Table 5). No effect on gene expression was observed after *T. gondii*

314 oocyst exposure.

315

316

317 **Table 4:** Immune marker responses after exposure to different stress at three conditions. Values are means  $\pm$  standard deviations ( $N = 10$ ). For each immune  
 318 parameter, and inside each stress, the difference between conditions is indicated by different letters with  $a > b > c$  ( $\alpha = 5\%$ ). Absence of letter means that no  
 319 significant difference has been observed between these conditions. PI+: percentage of cells labelled with propidium iodide; Casp+: percentage of cells  
 320 labelled with caspases 3/7 reagent; P.cap: percentage of cells that have ingested at least one microbead (phagocytic capacity); P.eff: percentage of cells that  
 321 have ingested at least three microbeads (phagocytic efficiency); P.nb: mean number of ingested microbeads (phagocytic avidity); NO+: NO basal production.  
 322

Immune marker		PI+ (%)		Casp+ (%)		P.cap (%)		P.eff (%)		P.nb		NO+ (FU)	
Stress	Condition	Mean $\pm$ SD	Diff	Mean $\pm$ SD	Diff	Mean $\pm$ SD	Diff	Mean $\pm$ SD	Diff	Mean $\pm$ SD	Diff	Mean $\pm$ SD	Diff
Cadmium chloride	0 $\mu$ g/L	16,0 $\pm$ 3,8	b	10,5 $\pm$ 1,9		53,0 $\pm$ 3,0		30,4 $\pm$ 3,3		5,9 $\pm$ 0,6		12125 $\pm$ 5453	
	25 $\mu$ g/L	21,9 $\pm$ 2,9	a	11,8 $\pm$ 2,8		54,7 $\pm$ 2,9		31,3 $\pm$ 3,1		5,8 $\pm$ 0,4		13821 $\pm$ 3481	
	250 $\mu$ g/L	22,2 $\pm$ 2,7	a	11,3 $\pm$ 2,8		54,5 $\pm$ 3,0		31,6 $\pm$ 3,5		6,0 $\pm$ 0,7		16230 $\pm$ 12251	
Ionomycin	0 $\mu$ M	19,4 $\pm$ 3,5	b	13,0 $\pm$ 6,2	b	42,3 $\pm$ 5,4	a	22,1 $\pm$ 4,1	a	5,8 $\pm$ 0,7	a	9414 $\pm$ 8966	
	1 $\mu$ M	19,4 $\pm$ 4,9	ab	16,7 $\pm$ 3,4	ab	38,8 $\pm$ 11,2	ab	19,3 $\pm$ 5,4	ab	5,3 $\pm$ 0,8	a	8835 $\pm$ 5408	
	10 $\mu$ M	25,4 $\pm$ 2,7	a	28,4 $\pm$ 6,4	a	32,5 $\pm$ 4,9	b	9,6 $\pm$ 3,4	b	2,8 $\pm$ 0,4	b	6955 $\pm$ 2753	
Ultraviolet B	0 min	17,2 $\pm$ 2,8	b	18,5 $\pm$ 3,1	b	50,8 $\pm$ 7,7		28,7 $\pm$ 8,6		6,7 $\pm$ 1,2	a	8028 $\pm$ 3151	
	3 min	22,9 $\pm$ 2,9	a	29,7 $\pm$ 3,1	a	46,8 $\pm$ 5,3		20,5 $\pm$ 4,4		4,9 $\pm$ 0,8	b	8596 $\pm$ 3011	
	10 min	25,9 $\pm$ 1,8	a	34,9 $\pm$ 3,3	a	47,3 $\pm$ 2,8		18,7 $\pm$ 3,1		4,4 $\pm$ 0,9	b	8874 $\pm$ 1062	
<i>Toxoplasma gondii</i>	0 : 1	12,8 $\pm$ 6,1		8,5 $\pm$ 1,4		65,4 $\pm$ 7,3	a	52,1 $\pm$ 8,1		6,6 $\pm$ 1,4	a	8162 $\pm$ 2240	b
	1 : 25	16,2 $\pm$ 6,6		8,6 $\pm$ 1,2		57,7 $\pm$ 6,7	ab	42,1 $\pm$ 14,3		5,5 $\pm$ 0,5	ab	12273 $\pm$ 5172	ab
	1 : 5	14,1 $\pm$ 5,8		8,6 $\pm$ 2,2		52,1 $\pm$ 8,1	b	33,6 $\pm$ 12,6		5,0 $\pm$ 1,0	b	18468 $\pm$ 8782	a
<i>Cryptosporidium parvum</i>	0 : 1	10,1 $\pm$ 3,0	b	15,1 $\pm$ 7,9		55,3 $\pm$ 8,0	a	30,7 $\pm$ 8,1	a	6,9 $\pm$ 1,8	a	9269 $\pm$ 2538	b
	1 : 1	13,4 $\pm$ 3,1	ab	15,3 $\pm$ 6,7		48,7 $\pm$ 10,1	ab	23,3 $\pm$ 10,0	ab	6,2 $\pm$ 1,5	ab	11387 $\pm$ 2522	ab
	5 : 1	14,1 $\pm$ 4,0	a	15,2 $\pm$ 6,2		40,1 $\pm$ 14,0	b	18,1 $\pm$ 10,1	b	4,9 $\pm$ 1,1	b	13111 $\pm$ 3039	a

323  
 324  
 325

326 **Table 5:** ddPCR results after exposure of hemocytes to different stress at three conditions. Values are means  $\pm$  standard error of the mean (N = between 8  
 327 and 10). For each measured parameter, and inside each stress, the difference between conditions is indicated by different letters with a > b > c ( $\alpha = 5\%$ ).  
 328 Absence of letter means that no significant difference has been observed between these conditions. *sod*: superoxide dismutase; *cat*: catalase; *gpx*:  
 329 glutathione peroxidase; *gst*: glutathione-S-transferase; *casp 3/7*: caspases 3/7; *arp 2/3*: actin-related protein 2/3; *cdc 42*: cell division control 42; *gal 2*: galectin

Molecular marker		<i>sod</i> (10-2 copies/hemocyte)		<i>cat</i> (10-2 copies/hemocyte)		<i>gpx</i> (10-2 copies/hemocyte)		<i>gst</i> (10-2 copies/hemocyte)		<i>mt</i> (10-2 copies/hemocyte)	
Stress	Condition	Mean $\pm$ SEM	Diff	Mean $\pm$ SEM	Diff						
Cadmium chloride	0 $\mu$ g/L	17.61 $\pm$ 2.63	ab	3.65 $\pm$ 0.69		4.32 $\pm$ 2.35		74.22 $\pm$ 9.09		797.03 $\pm$ 218.43	331
	25 $\mu$ g/L	23.78 $\pm$ 3.70	a	5.35 $\pm$ 0.65		3.16 $\pm$ 0.68		88.09 $\pm$ 18.86		1023.27 $\pm$ 482.92	
	250 $\mu$ g/L	8.84 $\pm$ 2.87	b	3.08 $\pm$ 0.61		2.28 $\pm$ 0.69		48.98 $\pm$ 19.99		679.67 $\pm$ 289.63	
Ionomycin	0 $\mu$ M	4.60 $\pm$ 1.44		2.46 $\pm$ 0.45		1.03 $\pm$ 0.67		13.44 $\pm$ 4.70		118.39 $\pm$ 58.39	
	1 $\mu$ M	4.13 $\pm$ 1.02		2.80 $\pm$ 0.57		1.65 $\pm$ 1.11		11.23 $\pm$ 4.73		125.91 $\pm$ 61.52	
	10 $\mu$ M	12.13 $\pm$ 6.10		2.98 $\pm$ 0.66		2.05 $\pm$ 0.63		33.91 $\pm$ 13.41		188.48 $\pm$ 74.39	
Ultraviolet B	0 min	13.95 $\pm$ 5.69		3.84 $\pm$ 0.82		2.01 $\pm$ 1.05		22.54 $\pm$ 2.16		876.73 $\pm$ 419.65	
	3 min	18.48 $\pm$ 4.64		3.66 $\pm$ 1.05		3.74 $\pm$ 1.00		53.85 $\pm$ 22.64		780.98 $\pm$ 254.24	
	10 min	17.28 $\pm$ 7.03		3.86 $\pm$ 0.82		2.86 $\pm$ 1.13		62.42 $\pm$ 33.06		472.30 $\pm$ 155.10	
<i>Toxoplasma gondii</i>	0 : 1	18.10 $\pm$ 5.78		4.70 $\pm$ 1.16		2.89 $\pm$ 0.91		14.02 $\pm$ 4.65		746.23 $\pm$ 311.75	
	1 : 25	12.46 $\pm$ 2.82		3.45 $\pm$ 0.38		2.03 $\pm$ 0.21		23.86 $\pm$ 6.13		542.38 $\pm$ 133.37	
	1 : 5	9.15 $\pm$ 2.03		2.83 $\pm$ 0.37		3.01 $\pm$ 0.26		21.51 $\pm$ 5.53		407.53 $\pm$ 111.06	
<i>Cryptosporidium parvum</i>	0 : 1	29.86 $\pm$ 14.99		5.75 $\pm$ 2.06		4.25 $\pm$ 1.40		80.42 $\pm$ 38.29		138.85 $\pm$ 51.56	
	1 : 1	44.85 $\pm$ 30.81		5.25 $\pm$ 0.91		9.07 $\pm$ 5.41		110.91 $\pm$ 67.98		350.18 $\pm$ 112.44	
	5 : 1	77.24 $\pm$ 32.27		11.64 $\pm$ 4.73		31.24 $\pm$ 26.51		176.15 $\pm$ 74.80		370.46 $\pm$ 88.04	

2.

Molecular marker		<i>casp 3/7</i> (10-2 copies/hemocyte)		<i>arp 2/3</i> (10-2 copies/hemocyte)		<i>cdc 42</i> (10-2 copies/hemocyte)		<i>gal 2</i> (10-2 copies/hemocyte)	
Stress	Condition	Mean $\pm$ SEM	Diff	Mean $\pm$ SEM	Diff	Mean $\pm$ SEM	Diff	Mean $\pm$ SEM	Diff
Cadmium chloride	0 $\mu$ g/L	4.61 $\pm$ 1.08		43.72 $\pm$ 24.10		5.54 $\pm$ 1.62		543.39 $\pm$ 243.64	
	25 $\mu$ g/L	4.95 $\pm$ 0.68		85.49 $\pm$ 22.86		4.75 $\pm$ 1.47		622.05 $\pm$ 309.40	
	250 $\mu$ g/L	4.17 $\pm$ 0.86		19.02 $\pm$ 3.02		4.82 $\pm$ 3.95		388.71 $\pm$ 204.43	
Ionomycin	0 $\mu$ M	1.87 $\pm$ 0.69		2.35 $\pm$ 2.17		0.67 $\pm$ 0.32		84.50 $\pm$ 49.09	
	1 $\mu$ M	2.17 $\pm$ 0.90		6.06 $\pm$ 5.91		0.52 $\pm$ 0.20		92.13 $\pm$ 50.47	
	10 $\mu$ M	4.51 $\pm$ 1.10		27.63 $\pm$ 19.16		0.91 $\pm$ 0.28		152.18 $\pm$ 58.40	
Ultraviolet B	0 min	8.88 $\pm$ 5.09		19.09 $\pm$ 18.60		1.38 $\pm$ 0.25		322.69 $\pm$ 214.44	
	3 min	7.15 $\pm$ 3.64		59.96 $\pm$ 39.44		3.60 $\pm$ 0.66		212.86 $\pm$ 147.02	
	10 min	3.01 $\pm$ 0.75		5.00 $\pm$ 3.73		0.62 $\pm$ 0.27		124.87 $\pm$ 92.37	
<i>Toxoplasma gondii</i>	0 : 1	2.55 $\pm$ 0.87		59.62 $\pm$ 26.00		7.28 $\pm$ 1.81		278.70 $\pm$ 64.58	
	1 : 25	2.59 $\pm$ 0.50		43.27 $\pm$ 13.72		5.55 $\pm$ 0.86		465.59 $\pm$ 82.17	
	1 : 5	2.16 $\pm$ 0.52		36.11 $\pm$ 10.35		4.82 $\pm$ 1.27		300.97 $\pm$ 70.90	
<i>Cryptosporidium parvum</i>	0 : 1	2.48 $\pm$ 0.86	b	52.78 $\pm$ 17.88		5.88 $\pm$ 0.41		371.40 $\pm$ 128.08	
	1 : 1	6.94 $\pm$ 1.84	ab	52.07 $\pm$ 11.35		4.42 $\pm$ 0.66		384.06 $\pm$ 135.49	
	5 : 1	9.48 $\pm$ 3.43	a	58.51 $\pm$ 9.05		3.35 $\pm$ 0.57		334.38 $\pm$ 58.13	

## 332 4. Discussion

### 333 4.1. Chemical stress induced different effects

334 Many authors have investigated the effects of cadmium on animals, including bivalves.  
335 These organisms are known to accumulate high doses of this metal, and thus are used as  
336 sentinel species for metallic biomonitoring [40, 41]. The effects of Cd on mussel physiology  
337 are numerous and complex, depending on the dose and bioavailability of metal as well as the  
338 size and reproductive status of mussels [41, 42]. Nonetheless, several effects on bivalve  
339 hemocytes are often highlighted by authors. Cadmium has deleterious effects on hemocyte  
340 viability, on the one hand being pro-apoptotic, and on the other hand by inducing necrosis  
341 [43-45]. This impact was observed in this study with an increase in necrosis following  
342 hemocyte exposure to 25 µg/L Cd and more, but no effect on hemocyte apoptosis (assessed  
343 by activation of caspases 3/7) was reported contrary to the literature [43-45]. Nevertheless,  
344 the conditions for exposing cells to cadmium were different. Also, Sokolova *et al.* [43] have  
345 reported the induction of apoptosis after exposure to Cd without caspase activation. Cd<sup>2+</sup>  
346 would induce apoptosis in a caspase-independent manner [46]. Disturbance of SOD  
347 production after exposure to Cd is a common feature, highlighted in our study and by several  
348 authors [44, 45, 47]. Cadmium can also alter immune functions in bivalves. Indeed, this metal  
349 can disturb cytoskeletal actin and thus modify adhesion and phagocytic process, leading  
350 sometimes to immune suppression [48-50]. No significant effect of Cd on *D. polymorpha*  
351 immune function was reported in this study, probably explained by several factors. First, Cd  
352 concentrations were higher than those of other studies with effects on immune functions [48,  
353 50]. Then, exposure conditions and duration were different (*ex vivo* versus *in vivo*), and  
354 finally the necrosis and phagocytosis double-labelling allowed to remove dead cells for  
355 phagocytic parameters. It should be noted that despite the importance of inter-pool  
356 variations, the highest concentration of cadmium seemed to decrease the expression of part  
357 of the genes studied. The expression levels of *sod*, *gpx*, *gst*, *arp 2/3* and *gal2* decreased at  
358 the highest concentration of Cd.

359 Results obtained after exposure of hemocytes to ionomycin are consistent with those of the  
360 literature. Ionomycin is a  $\text{Ca}^{2+}$  ionophore that increases intracellular  $\text{Ca}^{2+}$ , leading to pH  
361 modifications inside cells, such as hemocytes [51-54]. These effects induce the phenomenon  
362 of programmed death, named apoptosis. This was revealed in our study, with a dose-  
363 dependent activation of caspases 3/7 inside hemocytes and with an increase of *casp 3/7*  
364 gene expression, as revealed in several studies [51-53]. Ionomycin induces the intrinsic  
365 pathway of apoptosis by stimulating the activation of the initiator caspase 9 and executor  
366 caspases (caspases 3 and 7) as well as the release of cytochrome c from mitochondria, thus  
367 limiting the anti-apoptotic factors [52]. These authors highlighted that three hours after  
368 ionomycin exposure is enough to induce apoptosis and necrosis, as compared to our results  
369 [52, 55]. Few studies, however, have observed a decrease in phagocytosis after cell  
370 exposure to this antibiotic, contrary to our results. These decreases of phagocytic  
371 parameters assessed in mussel hemocyte can be explained by several hypotheses. First, it  
372 has been noticed that a part of phagocytosis can be  $\text{Ca}^{2+}$ -dependent, at least in human  
373 neutrophils [56-58].  $\text{Ca}^{2+}$  would act as a second messenger for phagocytosis and superoxide  
374 anion formation [59]. The disruption in intracellular transport caused by ionomycin may thus  
375 alter this immune function. Secondly, apoptotic hemocytes, rapidly ingested after ionomycin  
376 exposure [55], can be preferred to microbeads by viable hemocytes. Finally, although  
377 propidium iodide-labelled hemocytes (in necrosis) are removed from the phagocytosis assay,  
378 those labelled with caspase 3/7 reagent are not. The increase of apoptotic cells after  
379 exposure to ionomycin may directly lead to a decrease in phagocytosis of the microbeads.  
380 This hypothesis was supported by a tendency to increase the expression level of caspases  
381 3/7 when the ionomycin concentration rises up. This was also observed with other genes  
382 under study, such as *sod*, *gst*, *arp 2/3* and *gal2*, with induction factor compared to control  
383 condition from 2 to 11 for *arp 2/3*.

384 Cellular activities are coordinated by the information contained in genetic material. This  
385 creates a close link between gene expression and protein activity. However, many stages of  
386 gene regulation exist. In eukaryotes, they are found at the level of chromatin structure,

387 transcription, maturation of mRNAs and their temporal stability, at the level of translation and  
388 post-translational modifications that can also impact the activity of encoded protein [60]. In  
389 this study, no significant correlation between gene expression (*casp 3/7* on one side and *arp*  
390 *2/3*, *cdc 42* and *gal 2* on the other) and protein or cellular activity (activation of caspases 3/7  
391 and phagocytosis, respectively) had been observed. However, the comparison of the results  
392 obtained by flow cytometry and by ddPCR can provide information on the synergy of action  
393 of different studied stress. Indeed, the higher mortality rate (necrosis and apoptosis analysed  
394 by flow cytometry), caused by high concentrations of cadmium and ionomycin, could explain  
395 the modifications, although not significant, of the gene expressions (*sod* [ $p\text{-value} = 1.67 \times 10^{-2}$ ],  
396 *gst* and *arp 2/3* for cadmium at 250  $\mu\text{g/L}$ ; *sod*, *gst*, *casp 3/7*, and *arp 2/3* for ionomycin at  
397 10  $\mu\text{M}$ ). Both stress, at the highest intensities, had opposite effects. Cadmium tends to  
398 decrease gene expression while ionomycin would increase it (Figure 1). Despite a similar  
399 cytotoxic effect, this suggests that these two chemical stressors do not have the same  
400 modes of action. Cadmium is known to induce oxidative stress in cells and this oxidative  
401 stress would certainly impair the integrity of hemocytes. This could be the cause of the  
402 insignificance of the results in gene expression for the highest concentration of cadmium.  
403 Otherwise, activation of calcium channels ultimately leads to cell apoptosis by ionomycin  
404 further impacts immune cell functions and could induce the expression of genes linked to  
405 phagocytosis. Nevertheless, *arp 2/3*, *cdc 42* and *gal 2* have other functions than those  
406 related to phagocytosis. *cdc 42* and *arp 2/3* play roles in the cellular cycle and maintaining  
407 cellular integrity, while Gal 2 regulates the inflammation, cell homeostasis and cell growth  
408 processes [28, 61, 62].

409 Regarding caspases 3/7, no similarity of effect was observed between the results obtained in  
410 cytometry and in ddPCR. It is likely that the stress exerted on the hemocytes only influenced  
411 the inactive procaspases already present in the hemocytes without inducing modulation of  
412 their gene expression. Ionomycin, which induced significant activation of 3/7 caspases at the  
413 highest concentration, also seemed to induce the gene expression of these caspases.

414 However, this induction was just a trend. A larger number of biological replicas could improve  
415 the discernment of these effects.

416

#### 417 4.2. A cytotoxic physical stress

418 The physical stress, ultraviolet B, induced hemocyte necrosis and apoptosis as well as  
419 decrease of phagocytic avidity. These effects are in agreement with those reported by  
420 several studies. Clydesdale *et al.* [63] have synthesized effects of ultraviolet on human cells,  
421 such as apoptosis and necrosis induction and immunomodulatory effects. These effects have  
422 been also reported on aquatic invertebrates, such as crustaceans [64, 65], and bivalves [66,  
423 67]. Apoptosis caused by UVb light seems to be activated by the intrinsic way, *via* DNA  
424 fragmentation, caspases activation, modulation of cytoplasmic calcium, increase in  
425 mitochondrial membrane potential, and modifications of pro- and anti-apoptotic gene  
426 expression [64-67]. UV are thus immune toxic as well as genotoxic, as observed by Rank  
427 and Jensen [68] on hemocytes of *Mytilus edulis* with the same emission wave length (254  
428 nm).

429

#### 430 4.3. Different protozoa, different interactions?

431 Few studies are looking at protozoan:hemocyte interaction when the host is paratenic.  
432 Previously, we have described the effects of *C. parvum* and *T. gondii* oocysts on hemocytes  
433 of two bivalves, *D. polymorpha* and *M. edulis* [26]. The vast majority of effects previously  
434 observed have been also reported in this study. We observed a decrease of phagocytic  
435 parameters after exposure to both protozoa and an increase of basal NO production after  
436 exposure to *C. parvum* oocysts [26].

437 Decreases of phagocytosis can be explained by the establishment of immune process by  
438 hemocytes. Palos Ladeiro *et al.* [15] have reported that hemocytes of zebra mussels are able  
439 to engulf *C. parvum* oocysts. This protozoan has been used as a marker of phagocytosis for

440 *Crassostrea virginica* [69]. The *T. gondii* oocyst being about twice the size of that of *C.*  
441 *parvum*, it seems unlikely that it can be ingested. Nevertheless, immune cells of zebra  
442 mussels can aggregate around the oocysts to realize the phenomenon of encapsulation [70].  
443 These immune functions are accompanied by an overproduction of NO, which can be  
444 produced against microorganisms [71]. Not observed in previous studies [26, 70], oocysts of  
445 *C. parvum* induced hemocyte necrosis. Although the number of activated caspases 3/7 did  
446 not differ among the condition of *C. parvum* exposure, this protozoan induced gene  
447 expression of these enzymes. Terahara and Takahashi [72] have reported an over-  
448 expression of caspase genes as an immune response of mollusc host against parasite  
449 infection. Induction of apoptosis with parasitic exposure was often highlighted in edible  
450 bivalves [73-75]. If this exposure is prolonged, apoptosis can even be suppressed [75, 76].  
451 Nonetheless, these results were reported with specific hosts (i.e. *Crassostrea virginica* for  
452 *Perkinsus marinus*) and not for paratenic hosts. We previously evidenced that *T. gondii* was  
453 able to activate caspases 3/7 in hemocytes of the blue mussel [26]. Functional changes in  
454 the immunity of *Dreissena polymorpha* after exposure to both protozoa were more numerous  
455 than changes in the expression of genes linked to the immune system. *T. gondii* and *C.*  
456 *parvum* oocysts did not significantly impact immune gene expression in hemocytes of zebra  
457 mussels. Many authors have reported over-expression of genes following the exposure of  
458 molluscs to parasites, particularly lectins MTs or GSTs [77-81]. However, a big difference  
459 between these results and ours is that all of these studies were focused on a specific host,  
460 which could be subject to infection by the parasite. Recently, Leprêtre *et al.* [82] compared  
461 the protein expression profiles on the hemolymphatic compartments of zebra mussels  
462 between unexposed samples and others exposed to *C. parvum*. The changes in protein  
463 expression after exposure to this protozoan were mainly located at the cell level, and  
464 included proteins linked to cell motility, to the internalization and destruction of pathogens,  
465 apoptosis, and immune signalization, including an up-regulation of arp 2/3 complex, cdc 42  
466 and a galectin proteins, confirming our results obtained in flow cytometry. Nonetheless,  
467 Leprêtre *et al.* [82] emphasized the importance of the plasma compartment in the immune

468 responses of the zebra mussel. Some immune proteins have been up or down modulated  
469 after a 4-hour exposure to *C. parvum*, including arp 2/3, galectin, serine, clathrin and  
470 complement-like proteins. In our study, no correlation between the disturbances of  
471 phagocytosis by hemocytes exposed to both biological stressors and the expression of  
472 genes potentially linked to this process (*arp 2/3*, *cdc 42*, *gal 2*) has been reported. This can  
473 be explained by the fact that phagocytosis included several immune processes, such as  
474 vesicle transport, cell motility, cell adhesion, cytoskeleton organisation, etc., and by a large  
475 number of genes and proteins involved in this defence system [32, 83]. The complementarity  
476 between the two hemolymphatic compartments (plasma and hemocytes) is significant. Thus,  
477 the exposure to the various stresses carried out without plasma may block this  
478 complementarity, in particular for the recognition of pathogens [32]. It would be interesting to  
479 expose the hemolymph of the zebra mussels as a whole before separating plasma and cells.  
480 The cellular and molecular markers studied on hemocytes after this protocol may be different  
481 from those analysed without the plasma-hemocyte complementarity.

482

#### 483 4.4. Improvements and future tracks

484 It is important to note the high variability of gene expression among pools. This variability  
485 was reported between pools inside a condition (SEM) and between stress conditions (Table  
486 5). This variability was not identical according to the studied genes. Comparing gene  
487 expression for control conditions of each exposure, ratio of 2 (*cat*) to 30 (*arp 2/3*) were  
488 reported. Expression levels of *casp 3/7*, *cat*, *gal 2* and *mt* seemed to be less variable than  
489 those of *sod*, *gpx*, *gst*, *arp 2/3* and *cdc 42* genes. This strong variability can be explained by  
490 three hypotheses. The main reason for explaining this variation would be linked to  
491 interactions between hemocytes from different individuals within the same pool. We decided  
492 to work with pool to have enough hemocytes to expose the same sample and analyse all the  
493 markers on the same group. Barjhoux *et al.* [37] have observed a reduction in the variability  
494 of the immune markers analysed by flow cytometry in this species. However, the interaction

495 between hemocytes from different individuals may have caused this strong variability in  
496 molecular markers. Volume improvements on the hemolymph sample for this species would  
497 make it possible to analyse the expression of several genes individually.

498 Although the production of proteins in response to external stimuli comes from a rapid  
499 expression of genes, the regulatory steps induce a strong variation in the kinetics between  
500 gene expression and expression of the protein formed [84]. This is especially important when  
501 studying a biological process (i.e. phagocytosis), involving a large number of molecules [85].  
502 Thus, after exposure to a xenobiotic, the expression profiles vary depending on the post-  
503 exposure period at which analyses are carried out [86]. The kinetics of analysis can also  
504 modify our gene expression results. In our study, hemocytes were exposed for four hours  
505 before genetic material extraction. However, this duration may be too long to observe a  
506 modulation of gene expression. Morga *et al.* [80] highlighted over-expression of several  
507 genes (*gst*, *p450 cytochrome*, *galectin* and *lysozyme*) in hemocytes of *Ostrea edulis* after  
508 one hour *in vitro* exposure to *Bonamia ostreae*. These expressions were returned to basic  
509 levels 2 or 4 hours after exposure [80]. The kinetics of exposure (four hours exposure) could  
510 explain that the stress had more effects on the hemocyte functions than on the gene  
511 expression. On the other hand, this modulation of gene expression can appear later than four  
512 hours post-stress. Indeed, Farcy *et al.* [87] reported, in *C. gigas*, an over-expression of genes  
513 coding for stress proteins 4 to 8 hours after heat shock, this expression returning to basic  
514 levels after 48 hours. Correlatively, the expression of immune genes from the same bivalve  
515 (*C. gigas*) was stimulated after 24 to 48 hours after exposure to a virus (Ostreid herpesvirus  
516 1 mVar), and the return to baseline levels took place after 120 hours [88]. Nonetheless, these  
517 two studies were conducted *in vivo*. Although the 4-hours post-stress exposure period was  
518 established in previous work [26], this sampling time is ideal for cytometric analyses and to  
519 keep healthy cells. However, for the analysis of gene expression, it would be interesting to  
520 follow the kinetics of post-exposure expression over several hours to confirm our results.

521 These biases are mainly due to a narrow vision of the complex processes involved in the  
522 response to stress. In the last decade, the omics approaches (from genomics to

523 interactomics) permit to obtain the entire toxicological signature of a xenobiotic. These  
524 approaches would allow a larger view of the interaction between chemical, physical and  
525 biological stress and hemocytes. Even if the mechanisms which govern remains unclear,  
526 they are mainly controlled at the protein level (i.e. cytokines, immune recognition factors,  
527 etc.). The proteomic approach would answer several questions: i) do the reductions in  
528 phagocytosis parameters following exposure to biological stress and ionomycin have the  
529 same mechanisms? and ii) despite fairly similar cellular effects, are the oocysts of *T. gondii*  
530 and *C. parvum* managed in the same way by cell (recognition, destruction, etc.)? Finally,  
531 proteomics, flow cytometry and ddPCR could be performed jointly on proteins, activities and  
532 genes of interest previously chosen, during post-exposure kinetics to characterize the links  
533 between the different levels of organization.

534

## 535 **5. Conclusion**

536 One of the challenges of ecotoxicology today is to be able to link molecular biomarkers,  
537 cellular biomarkers and pathophysiological 'endpoints' with higher level ecological  
538 consequences [89]. This requires a good understanding of the effects of various xenobiotics  
539 as well as the responses of the organisms subjected to them. This study aimed to  
540 understand the immune response capabilities of zebra mussel hemocytes to various stresses  
541 (chemical, physical and biological), *via* cellular and molecular markers. Ultraviolet b are  
542 cytotoxic since they induced necrosis, apoptosis and reduced the phagocytosis of  
543 hemocytes. The oocysts from both protozoa induced nitric oxide production by exposed  
544 hemocytes and phagocytic parameters decreases, as already reported. Nonetheless,  
545 hemocytes appear to be more affected by oocysts of *C. parvum*, suggesting a difference in  
546 the management of these two protozoa. New gene sequences related to the immune system  
547 of zebra mussels were obtained in connection with apoptosis and cell motility and  
548 phagocytosis. Omics approaches could provide more details on the links between gene  
549 expression and cellular activity, particularly for complex processes such as phagocytosis. It

550 would be interesting to encompass the entire immune system by taking into account the  
551 plasma compartment. Indeed, plasma contains immune proteins which can play an important  
552 role in defence against xenobiotics [90, 91]. These two compartments could work together to  
553 eliminate some microorganisms [92]. The responses obtained on the entire hemolymphatic  
554 compartment may be different than those assessed exclusively at the cellular level.

555

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561

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