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1 **Comparison of viability and phagocytic responses of hemocytes withdrawn**
2 **from the bivalves *Mytilus edulis* and *Dreissena polymorpha*, and exposed to**
3 **human parasitic protozoa**

4

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15

16 **Abstract**

17 Bivalve molluscs are now considered indicator species of aquatic contamination by
18 human parasitic protozoa. Nonetheless, the possible effects of these protozoa on the
19 immune system of their paratenic hosts are poorly documented. The aim of this study
20 was to evaluate the effects of two protozoa on hemocyte viability and phagocytosis
21 from two mussels, the zebra mussel (freshwater habitat) and the blue mussel
22 (seawater habitat). For these purposes, viability and phagocytic markers have been
23 analysed on hemocytes from mussels without biological stress (control hemocytes),
24 and on hemocytes exposed to a biological stress (*Toxoplasma gondii* and
25 *Cryptosporidium parvum* oocysts). We report, for the first known time, the interactions
26 between protozoa and hemocytes of mussels from different aquatic environments.
27 Zebra mussel hemocytes showed a decrease in phagocytosis of fluorescent
28 microbeads after exposure to both protozoa, while blue mussel hemocytes reacted
29 only to *T. gondii* oocysts. These decreases in the ingestion of microbeads can be
30 caused by competition between beads and oocysts and can be influenced by the size
31 of the oocysts. New characterisations of their immune capacities, including
32 aggregation, remain to be developed to understand the specificities of both mussels.

33

34 *Keywords:* Immune markers; Hemocyte; Zebra mussel; Blue mussel; Protozoan

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39 1. Introduction

40 For several decades, new knowledge and techniques in the field of
41 ecotoxicology have raised awareness of environmental pollution and allows
42 environmental risk managers to act accordingly (Schwarzenbach et al., 2010).
43 However, many pollutants are still present in ecosystems, while new ones emerge
44 (Schwarzenbach et al., 2010), (Lauzent, M., 2017. Etude de l'écodynamique des
45 polluants organiques persistants et des micropolluants halogénés d'intérêt émergent
46 dans les milieux aquatiques. PhD Thesis, Université de Bordeaux, France).

47 Among these ubiquitous and persistent pollutants, protozoan parasites are of
48 particular interest for sanitary purposes (Conrad et al., 2005). Indeed, these protozoa
49 are responsible for a large and increasing number of waterborne outbreaks, i.e. the
50 cryptosporidiosis event in Milwaukee in 1993, affecting more than 400,000 people
51 (DeRegnier et al., 1989; Smith et al., 2006). Within this taxon, *Toxoplasma gondii* and
52 *Cryptosporidium* spp. are two parasites which can induce diseases with
53 complications in humans and animals, leading sometimes to host death (Conrad et
54 al., 2005; Gomez-Couso et al., 2006). Outside their hosts, these parasites are in their
55 environmental stage (oocyst), a long-lasting infective lifeform allowing the survival of
56 these organisms even when subjected to harmful external conditions (Palos-Ladeiro
57 et al., 2012). Thanks to their oocyst stage, these protozoa can survive for extended
58 periods in their environment, maintaining their infectivity (Tamburrini and Pozio, 1999;
59 Lindsay et al., 2003).

60 They are found in a lot of hydrosystems such as recreational water bodies,
61 rivers, drinking water, and upstream and downstream of the discharge points of
62 wastewater treatment plants (WWTPs), (Castro-Hermida et al., 2008; Helmi et al.,
63 2011; Kerambrun et al., 2016). Parasitic contamination of water has many origins, but
64 the main cause is runoff, concentrated with oocysts, from urban or agricultural areas
65 (from 1 to 1×10^5 cysts per liter for *Cryptosporidium parvum* (Lucy et al., 2008).
66 Precipitation events leading to the runoff of human or agricultural wastewater
67 contribute to the spread of these pathogens (Lucy et al., 2008). In coastal areas, this
68 can lead to contamination of the marine environment and the wildlife living there
69 (Gomez-Bautista et al., 2000; Shapiro et al., 2012). For example, *C. parvum* has
70 been found in the sea surrounding Hawaii as well as in the Mediterranean Sea
71 (Tamburrini and Pozio, 1999). This spread can be aggravated by the use of treated

72 wastewater, by adverse climatic conditions, or when demarcations between urban
73 and rural areas and wildlife habitats are narrow (Graczyk et al., 1997b; Conrad et al.,
74 2005; Castro-Hermida et al., 2008).

75 Furthermore, no treatment is effective in eliminating these oocysts in water.
76 Protozoa can pass through the treatment of wastewater due to their environmental
77 form being highly resistant to chemical and physical processes (Fayer et al., 1998;
78 Dumètre et al., 2013). In Europe and America, they are usually found in recreational
79 (swimming pools, fountains) and drinking waters at concentrations of up to 250
80 oocysts per liter, as reported by Smith et al. (2006). The waterway is not the only
81 source of contamination with these protozoa. Indeed, they can be captured by
82 paratenic hosts such as bivalve molluscs, potential prey of animals (i.e. sea otters)
83 and humans (Tamburrini and Pozio, 1999; Conrad et al., 2005). For all of these
84 reasons, *T. gondii* and *C. parvum* present a high health risk for both fauna and
85 humans.

86 At the present time, several studies have reported interest in molluscs as
87 indicators of parasitic loads in water, and the purpose of this method is to perform
88 direct assessment in water (Palos-Ladeiro et al., 2012; Shapiro et al., 2014). Indeed,
89 the filter feeders can accumulate parasites from their habitat, and this accumulation
90 seems to be proportional to the intensity of the parasitic contamination in water
91 (Graczyk et al., 2003; Palos-Ladeiro et al., 2014). While the water is constantly in
92 motion, bivalves are generally fixed, providing a better representation in the
93 assessment of parasitic load (Palos-Ladeiro et al., 2012). The wide range of indicator
94 species, present along the freshwater-marine water continuum, constitutes a final
95 advantage in the detection of parasites via molluscs. Among these species, the zebra
96 mussel (Graczyk et al., 2003; Lucy et al., 2008; Palos-Ladeiro et al., 2014), the blue
97 mussel (Lucy et al., 2008), the *Gammarus* (Bigot-Clivot et al., 2016), the Asian
98 freshwater clam (Graczyk et al., 2003), and oysters (Fayer et al., 1998; Esmerini et
99 al., 2010), are commonly used. Despite the increasing use of these molluscs as
100 indicators of aquatic protozoan contamination, knowledge of the effects of these
101 parasites on the health of their paratenic host is very limited. Previous studies have
102 reported the ability of bivalves to phagocytize some of these enteroprotezoa (Abbott
103 Chalew et al., 2012; Palos Ladeiro et al., 2018b), and we reported a cytotoxic effect
104 of these oocysts on zebra mussels, *Dreissena polymorpha* (Le Guernic et al., 2019).

105 Following this observation, we aim to enrich knowledge on the protozoan-
106 hemocyte interaction of bivalves representing the freshwater-marine water
107 continuum. Hemocytes, hemolymphatic circulating cells, play several roles in
108 physiological functions of invertebrates including nutrition, reproduction, detoxification
109 and immune capacities (Evariste et al., 2016). The study of the immune system is of
110 great interest since it can reveal the state of health of an organism (protection of the
111 organism against foreign constituents, maintenance of homeostasis, etc.), and the
112 immune system is the main barrier to microorganisms and parasites (Bols et al.,
113 2001; Salo et al., 2005).

114 The main aim of this study was to evaluate the immune responses of two
115 mussels, from different aquatic habitats, exposed to *T. gondii* or to *C. parvum*
116 oocysts. Beforehand, the comparison of basal levels of their immune markers was
117 necessary.

118 The two bivalves chosen, representative of two distinct aquatic environments,
119 are the blue mussel *Mytilus edulis* (sea water), and the zebra mussel *D. polymorpha*
120 (freshwater). The commercial interest of the blue mussel, its homology with the zebra
121 mussel for ecotoxicological studies (Binelli et al., 2015), their tolerance and plasticity
122 towards pollution (Binelli et al., 2015), their frequent use in biomonitoring programs
123 (i.e. Mussel Watch Program in USA, Besse et al. (2012)), and their ability to
124 accumulate (oo)cysts (Lucy et al., 2008), argue in favour of this choice.

125

126 **2. Materials and methods**

127 *2.1. Biological models*

128 *2.1.1. Zebra and blue mussels*

129 Two hundred and ten freshwater mussels were collected on 25th August 2017
130 from Der Lake (51290 Giffaumont-Champaubert, France, 48° 33' 35" N; 4° 45' 11" E),
131 while the same number of marine mussels was obtained from the Channel Sea
132 (76111 Yport, France, 49° 44' 41" N; 0° 18' 33" E) on 19th October 2017. Each
133 bivalve species was brought back from these sites to the URCA (Université de Reims
134 Champagne-Ardenne (URCA), France) laboratory and was acclimatised over several
135 weeks before the beginning of experiments. Freshwater (Cristaline Aurele drinking
136 water; spring Jandun, France, 6 L per tank) was maintained at 14.46 ± 0.72 °C; pH
137 7.51 ± 0.08 ; 550 ± 50 µS/cm, while reconstituted seawater (osmotic water + marine

138 sea salt; Tetra, Germany, 12 L per tank) was retained at 13.37 ± 0.46 °C; pH $7.87 \pm$
139 0.04 ; 61.31 ± 1.34 mS/cm; 31.53 ± 0.82 psu. During all acclimation and experimental
140 steps, two-thirds of the water volume were renewed twice each week to ensure the
141 total depuration of organisms. *Dreissena polymorpha* (28.3 ± 2.9 mm; 3.3 ± 0.5 g, 70
142 mussels per tank) were fed twice per week, with two microalga species,
143 *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, at a ratio of one million of each
144 species per mussel per day. *Mytilus edulis* (43.1 ± 3.1 mm; 15.5 ± 2.4 g, 70 mussels
145 per tank) were fed at the same intervals as zebra mussels, with *Isochrysis galbana*
146 algae at a ratio of four million cells per day per mussel. Four zebra mussels and one
147 blue mussel died during acclimation or experimental steps (1.9% and 0.5%,
148 respectively).

149

150 2.1.2. Hemolymph collection

151 In order to simplify the exposure of hemocytes to protozoa and to limit
152 exposure biases (adsorption, differential accumulation, etc.), these biological
153 stresses were applied during ex vivo exposures. Hemolymph recovery was done
154 before feeding in order not to threaten cytometric analyses. Furthermore, in order to
155 limit the individual variations of the cellular markers and to expose the same
156 individuals to the different concentrations of protozoa, hemolymph samples were
157 pooled. Consequently, hemolymph from five mussels of each species was withdrawn
158 from the posterior adductor muscle with 0.3 mL syringes (30 G x 8 mm, Becton
159 Dickinson, NJ, USA) for zebra mussels and 1 mL syringes (23 G x 1", Becton
160 Dickinson) for blue mussels. Samples were kept on ice and pooled in order to obtain
161 enough hemolymph volume for all flow cytometric analyses. Cell counts were
162 examined with KOVA slides (KOVA international, CA, USA) and cell concentrations
163 were adjusted for the two mussels and for each exposure condition to 375,000 cells
164 per microplate well (U-bottom, VWR, PA, USA). As hemocytes are adherent cells
165 (Delaporte et al., 2003; Labreuche et al., 2006), a sedimentation step was then
166 conducted according to internal protocols (Barjhoux et al., unpublished data) and
167 plasma was removed in order to retain only viable hemocytes for the experiment.
168 Then, hemocytes from zebra mussels were suspended in Leibovitz 15 medium (L15,
169 Sigma-Aldrich, MO, USA), modified according to Quinn et al. (2009) and Evariste et
170 al. (2016) protocols. For blue mussels, hemocytes were suspended in a marine

171 physiological saline solution (MPSS, pH 7.8, filtered with 0.2 µm filter, NaCl at 470
172 mM, KCl at 10 mM, CaCl₂ at 10 mM, Hepes at 10 mM, MgSO₄ at 47.7 mM, (Le Foll
173 et al., 2010; Rioult et al., 2014)).

174

175 2.2. Exposures to biological stress and assessment of immune parameters

176 Tested concentrations of *T. gondii* (generously gifted by J.P. Dubey, USDA
177 (U.S. Department of Agriculture), Beltsville, USA; strain ME-49 genotype II) and *C.*
178 *parvum* (INRA, Institut national de la recherche agronomique, Val de Loire research
179 center, France; Iowa isolate) oocysts were chosen in accordance with previous work
180 (Le Guernic et al., 2019), i.e. a ratio of protozoa:hemocytes of 1:25 and 1:5 for *T.*
181 *gondii* (15,000 and 75,000 oocysts per microplate well, respectively) and 1:1 and 5:1
182 for *C. parvum* (375,000 and 1,875,000 oocysts per microplate well, respectively).
183 Hemocytes were ex vivo exposed to these oocysts for 4 h (16 °C, in the dark).
184 Autofluorescence of *T. gondii* oocysts (UV), different from that of hemocytes, allows
185 us to remove them from flow cytometric analyses (Supplementary Fig. S1).
186 *Cryptosporidium parvum* oocysts are small enough not to be confused with
187 hemocytes of the two mussels, and they are therefore easily separable from
188 hemocytes during flow cytometry analyses (Supplementary Fig. S1).

189 To assess immune parameters, each pool exposed or not to biological stress
190 was immediately incubated for 4 h according to the following conditions: i) 2 µm
191 yellow-green fluorescent latex microbeads with a cell:bead ratio of 1:50 (4 h of co-
192 exposure to protozoa and beads at once, 16 °C, in the dark, Polysciences Inc., PA,
193 USA, (Barjhoux et al., unpublished data), ii) 0.5 µM caspases 3/7 green flow
194 cytometric reagent (30 min before the end of 4 h of protozoan-hemocyte exposure,
195 16 °C, in the dark, Thermofisher Scientific, MA, USA), and iii) 5 µM 4-amino-5-
196 methylamino-2',7'-difluorofluorescein diacetate (DAF-fm, 30 min before the end of 4 h
197 protozoan-hemocyte exposure, 16 °C, in the dark, Thermofisher Scientific). Ten
198 minutes before analyses, cells were removed into new wells with an anti-aggregate
199 solution (Trypsine-EDTA solution for *D. polymorpha*, Sigma-Aldrich, (Yang et al.,
200 2002) adapted on hemocytes of zebra mussel by Barjhoux et al. (unpublished data);
201 and Alsever solution for blue mussels (ALS, NaCl at 300 mM, Glucose at 100 mM,
202 Citrate sodium at 30 mM, citric acid at 26 mM, and EDTA at 10 mM, pH 5.4 (Rioult et
203 al., 2014)). Finally, cells were labelled with propidium iodide (PI, 1 % final, Sigma-

204 Aldrich) in order to reveal dead hemocytes. A control for each pool (without protozoan
205 and flow cytometry reagent) was performed in order to ensure that 4 h incubation did
206 not alter hemocyte viability/phagocytosis (data not shown).

207 Data from 10,000 cells per sample were counted for each suspension sample
208 with an Accuri™ C6 SORP flow cytometer (Becton Dickinson, N.J., USA, with BD
209 Accuri™ C6 software (v1.0.264.21)). Viability and phagocytosis markers, and their
210 corresponding method of analysis, are described in Table 1.

211

212 *2.3. Statistical analyses*

213 Statistical analyses were performed with R software (3.4.1). Comparisons
214 between basal levels of markers of both mussels were assessed with Student t or
215 Wilcoxon tests, according to results obtained with Shapiro-Wilk (distributions
216 normality) and Fisher (homoscedasticity) tests ($\alpha = 5\%$). For data of responses to
217 oocysts, an ANOVA was performed ($\alpha = 5\%$). ANOVA residuals normality was tested
218 with a Shapiro-Wilk test as well as variance homogeneity by a Levene test. If these
219 conditions were met, ANOVA results were kept, if not, a Kruskal-Wallis test was
220 performed ($\alpha = 5\%$). Then, a post-hoc test was performed in order to discriminate
221 significant differences within each group with a Tukey's HSD test (honestly significant
222 difference) with Holm correction (parametric data) or a Nemenyi test (non-parametric
223 data). This statistical treatment was used on 16 pools for basal differences between
224 mussels and on eight pools for responses to oocyst data.

225

226 **3. Results and discussion**

227 *3.1. Basal levels of immune parameters between hemocytes from *D. polymorpha* and* 228 **M. edulis**

229 Basal levels of immune markers are described in Table 2. Viability of
230 hemocytes from blue mussels was greater than that of zebra mussels, characterised
231 by lower hemocyte mortality (PI labelled hemocytes) and hemocyte apoptosis (cells
232 with activated caspases 3/7) ($P = 6.65 \times 10^{-6}$ and $P = 1.11 \times 10^{-4}$, respectively; Table
233 2). While hemocytes from the two bivalves ingested the same number of microbeads,
234 zebra mussel hemocytes had a phagocytic capacity and efficiency lower than those
235 of blue mussels (15% less on average, $P = 8.94 \times 10^{-6}$ and $P = 8.05 \times 10^{-6}$,

236 respectively; Table 2). No difference in basal NO (nitric oxide) production was
237 observed between zebra mussel and blue mussel hemocytes (Table 2).

238 Differences in basal levels of immune parameters between bivalve species
239 have been reported many times in the literature. Indeed, Wootton et al. (2003)
240 compared immune systems of three bivalves, *Mytilus edulis*, *Cerastoderma edule*,
241 and *Ensis siliqua*, and found great disparities between species. Percentages of
242 phagocytic hemocytes varied from 20 to 80% according to the species, as well as
243 phagocytic avidity, which varied from 4 to 10 ingested beads. Despite differences in
244 phagocytic capacity and efficiency (Table 2), our results suggest that *D. polymorpha*
245 and *M. edulis* are able to ingest the same number of microbeads. Important
246 variations in the immune parameters related to the different species have been
247 reported by Sauvé et al. (2002). In their study, immune markers of eight marine
248 bivalves (*Cyrtodaria siliqua*, *Mactromeris polynyma*, *Mesodesma arctatum*, *Mya*
249 *arenaria*, *Mya truncata*, *Mytilus edulis*, *Serripes groenlandicus*, *Siliqua costada*) and
250 two freshwater bivalves (*Dreissena polymorpha* and *Elliptio complanata*) were
251 compared. Phagocytic levels between these species varied from approximately 10%
252 to 60%, highlighting the high inter-species variation. In addition to these differences
253 in phagocytosis, the hemocyte mortality of these molluscs has been assessed
254 (Sauvé et al., 2002). After a longer incubation period (24 h), hemocytes presented a
255 mean necrotic level of approximately 26% (Sauvé et al., 2002). Hemocyte mortality
256 (PI-labelled cells) in zebra mussel hemolymph assessed in our study ($11.53 \pm 2.92\%$)
257 was lower than that found with the same species by Sauvé et al. (2002), and close to
258 the 16.37% reported by Evariste et al. (2016). Correlatively, blue mussel mortality
259 ranged from to 1.4 to 6.9%, and was comparable to that observed by Galimany et al.
260 (2008) (approximately 2.0%). As for NO production in our study, Wootton et al.
261 (2003) did not find significant differences of intracellular ROS (reactive oxygen
262 species) production between bivalve species. Nonetheless, these authors noticed
263 differences between species for extracellular ROS production. Given all the basal
264 differences in immune markers between zebra and blue mussels, it would have been
265 interesting to evaluate the extracellular NO production. Other studies have
266 highlighted basal differences between bivalve species, whether on phagocytosis
267 parameters (Ordas et al., 1999; Delaporte et al., 2003; Hurtado et al., 2011; Dang et
268 al., 2012), hemocyte mortality (Hurtado et al., 2011), oxidative activities (Gestal et al.,

269 2008; Hurtado et al., 2011), or other immune markers such as phenoloxidase activity
270 (Munoz et al., 2006) or lysosomal activity (Suresh and Mohandas, 1990).

271 There could be other explanations for the basal level differences observed
272 between species. First, conservation and resuspension buffers were necessarily
273 different between our two models. Although these buffers were tested and approved
274 by several authors (Quinn et al., 2009; Le Foll et al., 2010; Rioult et al., 2014;
275 Evariste et al., 2016; Barjhoux et al., unpublished data), this buffer difference could
276 modify basal biomarker levels. Indeed, the percentage of phagocytic cells of *M.*
277 *arenaria* were modified according to buffer solution and/or incubation time (Sauvé et
278 al., 2002). In our study, except for buffers, the ex vivo experimental procedures on
279 hemocytes of both mussels were strictly identical in order to simplify and allow
280 comparison. Secondly, since the plasma has been removed from samples, we did
281 not study the immune responses as a whole but only the cellular compartment.
282 Indeed, several immune and humoral factors are inside plasma including lectins and
283 antimicrobial peptides, which allow the recognition of non-self, opsonisation, the
284 recruitment of other hemocytes, or the immobilization of pathogens, or even their
285 degradation (Kim et al., 2006; Allam and Raftos, 2015). These humoral activities,
286 such as the proportion of lectins produced, can differ between bivalve species
287 (Wootton et al., 2003). The focus of intracellular responses may accentuate or
288 attenuate the differences in immune performance between our two biological models.

289 This study of basal immune marker levels between two mussels from two
290 aquatic habitats highlighted some dissimilarities. Since these differences have been
291 shown, it will be easier to compare their immune responses after exposure to various
292 stresses.

293

294 3.2. Effects of human protozoa on immune parameters of mussels

295 Similar to previous results obtained on zebra mussel hemocytes (Le Guernic
296 et al., 2019), exposure of these immune cells to *T. gondii* or *C. parvum* oocysts did
297 not impact their viability, whether for cell mortality (IP+) or apoptosis (Table 3).

298 On the contrary, hemocytes from blue mussels were impacted by *T. gondii* oocysts,
299 translated as dose-dependent increases in hemocyte mortality and apoptosis ($P =$
300 2.25×10^{-2} and $P = 2.50 \times 10^{-2}$ compared with control conditions, respectively; Table
301 3). *Toxoplasma gondii* oocysts, at the highest concentration (1:5), induced decreases

302 of phagocytic capacity and efficiency of hemocytes of both mussels (blue mussel: $P =$
303 4.46×10^{-4} and $P = 1.24 \times 10^{-3}$ compared with control conditions, respectively; zebra
304 mussel: $P = 6.10 \times 10^{-6}$ and $P = 2.24 \times 10^{-5}$ compared with control conditions,
305 respectively; Table 3, Fig. 1). Moreover, exposure of zebra mussel hemocytes to the
306 two biological stresses at the highest concentration induced ingestion of fewer
307 microbeads ($P = 3.00 \times 10^{-2}$ after *C. parvum* exposure and $P = 8.60 \times 10^{-3}$ after *T.*
308 *gondii* exposure). This effect was not observed in blue mussel hemocytes (Table 3).

309 The basal NO production seems to increase with protozoa concentrations, for
310 both protozoa and for both mussels (Table 3). Nevertheless, only hemocytes from
311 blue mussels exposed to *T. gondii* oocysts and those of zebra mussels exposed to *C.*
312 *parvum* oocysts produced a gradual and significant increase in intracellular NO ($P =$
313 2.25×10^{-2} between 0:1 and 1:5 conditions and $P = 3.91 \times 10^{-2}$ between 0:1 and 5:1
314 conditions, respectively, Table 3).

315 It has been reported many times that *M. edulis* and *D. polymorpha* can
316 accumulate *T. gondii* and *C. parvum* (Lucy et al., 2008; Palos-Ladeiro et al., 2015;
317 Kerambrun et al., 2016). Probably because mussels are paratenic hosts for these
318 protozoa, very few studies have investigated the possible effects of these parasites
319 on the mussels' immunity. Abbott Chalew et al. (2012) used *C. parvum* oocysts to
320 assess phagocytic levels of eastern oyster hemocytes (*Crassostrea virginica*). These
321 hemocytes were able to ingest from 10 to 30 oocysts, in a dose-dependent manner.
322 The corbicula, *Corbicula fluminea*, also ingested cysts of another enteroprotezoan,
323 *Giardia duodenalis* (Graczyk et al., 1997a). Correlatively, previous studies have
324 shown that zebra mussel hemocytes were able to engulf *C. parvum* oocysts (Palos
325 Ladeiro et al., 2018a), and were altered and able to initiate the encapsulation process
326 after exposure to *T. gondii* oocysts (Le Guernic et al., 2019). In our study, decreases
327 in phagocytosis of microbeads could be due to the establishment of immune
328 responses to parasites. Hemocytes which ingest protozoa, or which aggregate to
329 achieve encapsulation, certainly cannot ingest as many microbeads as unexposed
330 hemocytes. Other studies, focusing on the effects of protozoa on mussels, have
331 mainly studied *Perkinsus*, *Bonamia*, *Marteila*, or *Haplosporidium* spp., for which
332 mussels are specific hosts (Zannella et al., 2017). Effects of these protozoa on
333 hemocytes are varied and summarized by Zannella et al. (2017) and Soudant et al.
334 (2013). All immune components can be impacted, such as THC (total hemocyte
335 count) (Anderson et al., 1995; Soudant et al., 2013), ROS production (Anderson et

336 al., 1995; Soudant et al., 2013), phagocytosis (Goedken et al., 2005; Soudant et al.,
337 2013), apoptosis (Goedken et al., 2005; Soudant et al., 2013), lectin synthesis (Kim
338 et al., 2006; Soudant et al., 2013), etc. Immune system cellular components of
339 bivalves are impacted by numerous protozoan parasites, whether their hosts are
340 specific or paratenic.

341 Hemocytes of both mussels do not react similarly to both protozoa. Indeed,
342 while *T. gondii* and *C. parvum* induce almost the same effects on zebra mussels'
343 hemocytes (decreases in phagocytic parameters), blue mussels' hemocytes react
344 only to *T. gondii* oocysts (decreases in hemocyte viability and phagocytic
345 parameters). A principal component analysis (PCA) was performed on immune
346 reactions to protozoa for both mussels (Fig. 2). For the zebra mussel, the first axis,
347 separating exposure conditions, explains 45.42% of total inertia, and strong
348 contributions for this axis (> 20 %) are due to phagocytic capacity, efficiency and
349 avidity (Fig. 2). On the contrary, *M. edulis* markers show no difference in responses
350 between the control and *C. parvum* oocyst exposures (overlapped circles; Fig. 2),
351 while responses to *T. gondii* oocyst exposure are detached from other responses
352 according to both axes (49.89 and 29.35% of total inertia, respectively; Fig. 2). These
353 axes are built with a strong contribution of hemocyte mortality and phagocytic
354 capacity and efficiency for PC1, and by the phagocytic avidity and NO basal
355 production for PC2.

356 Immune responses against a biological stress depend on its nature but also on
357 bivalve species (Pruzzo et al., 2005). Our differences in responses to protozoa
358 according to mussel species are not surprising, and could be explained by several
359 hypotheses.

360 Firstly, species can have different immune abilities as well as different
361 specificities to pathogens. Few studies have been concerned with the effects of a
362 protozoan on at least two bivalve species. An exposure to *Perkinsus marinus* induced
363 higher phagocytosis in hemocytes of *C. virginica* than that of *C. gigas* (La Peyre et
364 al., 1995). Inversely, secretory products of *Perkinsus atlanticus* had reduced
365 phagocytosis of zymosan particles by bivalves' hemocytes, but was more
366 pronounced in mussels (*Mytilus galloprovincialis*) than in clams (*Ruditapes*
367 *decussatus*) (Ordas et al., 1999). *Perkinsus marinus* also can alter ROS or reactive
368 oxygen intermediates (ROI) production according to host species (Volety and Chu,
369 1995; Gestal et al., 2008). Bivalve species can react differently to other biological

370 stresses such as bacteria (Lambert and Nicolas, 1998; Allam et al., 2006) or protists
371 (Ford et al., 1993).

372 Secondly, these differences in responses could be explained by the
373 dissimilarities in the composition of the oocyst wall between *T. gondii* and *C. parvum*,
374 as well as by possible divergence in recognition of instigators between the two
375 mussels. Although oocyst walls of both protozoa have similarities such as the
376 presence of two layers providing resistance to mechanical and chemical stresses or
377 the presence of acid-fast lipids such as triglycerides, the wall of the *Toxoplasma*
378 *gondii* oocyst seems more sophisticated (Bushkin et al., 2013; Dumètre et al., 2013).
379 Indeed, this oocyst wall, contrary to that of *C. parvum*, contains dityrosine proteins,
380 allowing robustness and UV fluorescence, porous scaffold in the inner layer of the
381 oocyst wall constituted by fibrils of β -1,3-glucan, as well as more proteins (Bushkin et
382 al., 2013; Samuelson et al., 2013). This difference in composition may result in
383 different recognition by hemocytes. Indeed, several studies have highlighted the
384 presence of pattern recognition receptors (PRRs) specific to β -1,3-glucan in bivalves
385 (Itoh et al., 2010; Anderson et al., 2011). This recognition can lead to various immune
386 responses by hemocytes such as modifications of the distribution and number of
387 hemocytes, their adhesion / aggregation capacities, as well as ROS or reactive
388 nitrogen species (RNS) production (Costa et al., 2009; Anderson et al., 2011).
389 Hemocyte response differences of both mussels to protozoa may be related to the
390 presence and nature of these PRRs. In addition to these PRRs, some antimicrobial
391 peptides (AMPs) could have anti-protozoan activities (Reddy et al., 2004). Despite
392 new advances (Leprêtre et al., 2019), knowledge of AMPs and PRR in the blue
393 mussel is much more advanced than in the zebra mussel (Mitta et al., 2000; Reddy et
394 al., 2004; Song et al., 2010). Differences in PRRs and AMPs (nature and abundance)
395 between mussels could explain the response differences in these two bivalves to
396 protozoa.

397 Thirdly, as already discussed in section 3.1, the entire immune system has not
398 been taken into account because humoral factors were not assessed. Without
399 humoral factors, the recognition of oocysts by hemocytes can only be done directly
400 (Allam and Raftos, 2015). The proportion of cellular and humoral responses can vary
401 depending on the species (Wootton et al., 2003), which might explain the difference
402 in immune responses between the two bivalves.

403 The fourth and last hypothesis that could explain these differences is related to
404 the size of the two oocysts. The size of *T. gondii* oocysts is between 10 and 12 µm,
405 while that of *C. parvum* is approximately 4-5 µm (Abbott Chalew et al., 2012;
406 Dumètre et al., 2013). This difference in size might be important in the immune
407 strategy put in place by the hemocytes (phagocytosis or encapsulation). Indeed,
408 hemocytes are unable to phagocytose large particles and can implement
409 encapsulation to remove those (Cheng and Rifkin, 1970; Pila et al., 2016). We have
410 previously shown the setup of the encapsulation of *T. gondii* oocysts by the zebra
411 mussel hemocytes (Le Guernic et al., 2019), as well as the phagocytosis of *C.*
412 *parvum* oocysts by these hemocytes (Palos Ladeiro et al., 2018a). The hemocyte
413 responses to protozoa might therefore vary according to the size of the oocysts.

414 Although phagocytosis of microbeads decreased after exposure of both
415 mussels to protozoa, we have reported different immune capacities according to
416 mussel and oocyst species. New characterisations of their immune capacities,
417 including aggregation, remain to be developed to understand the specificities of both
418 mussels.

419

420 **4. Conclusion**

421 In this study, we proposed the evaluation of effects of protozoa on
422 hemocytes of two mussel species often used in environmental biomonitoring, taking
423 into account the basal differences in their immune responses. Without any stress,
424 cellular components of the blue mussel immune system are healthier and more active
425 than those in the zebra mussel. Mussels' hemocytes which were exposed to *T. gondii*
426 and *C. parvum* oocysts exhibited different immune responses. These responses
427 varied depending on the nature of the mussels and oocysts; while zebra mussels'
428 hemocytes reacted similarly to both oocysts, the blue mussel ones were more
429 impacted by *T. gondii* oocysts but not by *C. parvum*.

430 Characterisation of basal levels of markers of these two biological models
431 allows the comparison between these species and thus the possibility to perform
432 large scale biomonitoring (freshwater – marine water continuum). Nonetheless,
433 differences in impact and immune responses according to mussel species and
434 biological stresses, highlighted the necessity to continue study of their immune
435 characterisation. Consideration of the entire immune system via the study of humoral

436 factors contained in the plasma, the study of other immune markers (hemocyte
437 aggregation, motility, etc.), and in vivo experiments could improve our knowledge
438 about immune abilities and specificities of hemocytes from these mussels, and could
439 finally reduce comparison obstacles during large scale biomonitoring.

440

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452

453

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689

690 **Figure legends**

691

692 **Fig. 1.** Phagocytic efficiency (in percentage) according to mussel species and
693 conditions of hemocyte exposure to protozoa. X axis shows ratios of
694 protozoan:hemocyte. NS, non-significant. An asterisk represents a statistical and
695 significant difference between hemocytes exposed to different concentrations of one
696 protozoan (ANOVA or Kruskal-Wallis tests), with * $0.05 > P \geq 0.01$; ** $0.01 > P \geq$
697 0.001 ; and *** $P < 0.001$ on $n=8$.

698

699 **Fig. 2.** Principal component analyses (PCAs) representing individuals grouped
700 according to protozoan exposure (illustrative variable). (A) PCA built with results
701 obtained from *Dreissena polymorpha*. (B) PCA built with results obtained from *Mytilus*
702 *edulis*. PC1 and PC2 are constructed from all immune markers analysed, with two
703 selected dimensions. Percentages before parentheses are the inertia followed by
704 eigenvalues for each axis. Table below summarizes the contributions to the axes of
705 each variable (in percentage). PI, hemocyte mortality; P.cap, phagocytic capacity;
706 P.eff, phagocytic efficiency; P.nb, phagocytic avidity; NO, nitric oxide basal
707 production; Casp, hemocyte apoptosis.

708

709 **Supplementary Fig. S1.** Flow cytometry graphs explaining how the oocysts of
710 protozoa (*Toxoplasma gondii* and *Cryptosporidium parvum*) were removed from the
711 analyses. SSC, side-scattered light (complexity); FSC, forward-scattered light (size);
712 FL, fluorescence. The red dotted gate specifies the events recorded for the analyses.

713

Fig. 1

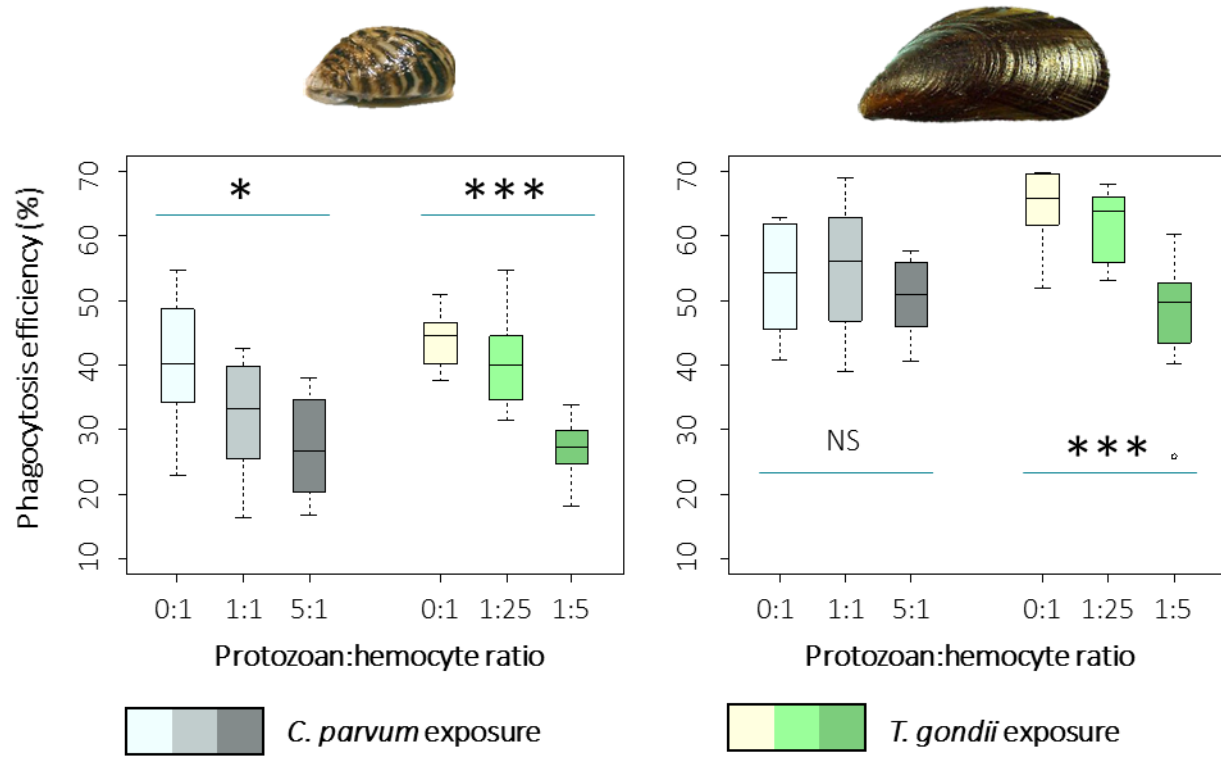
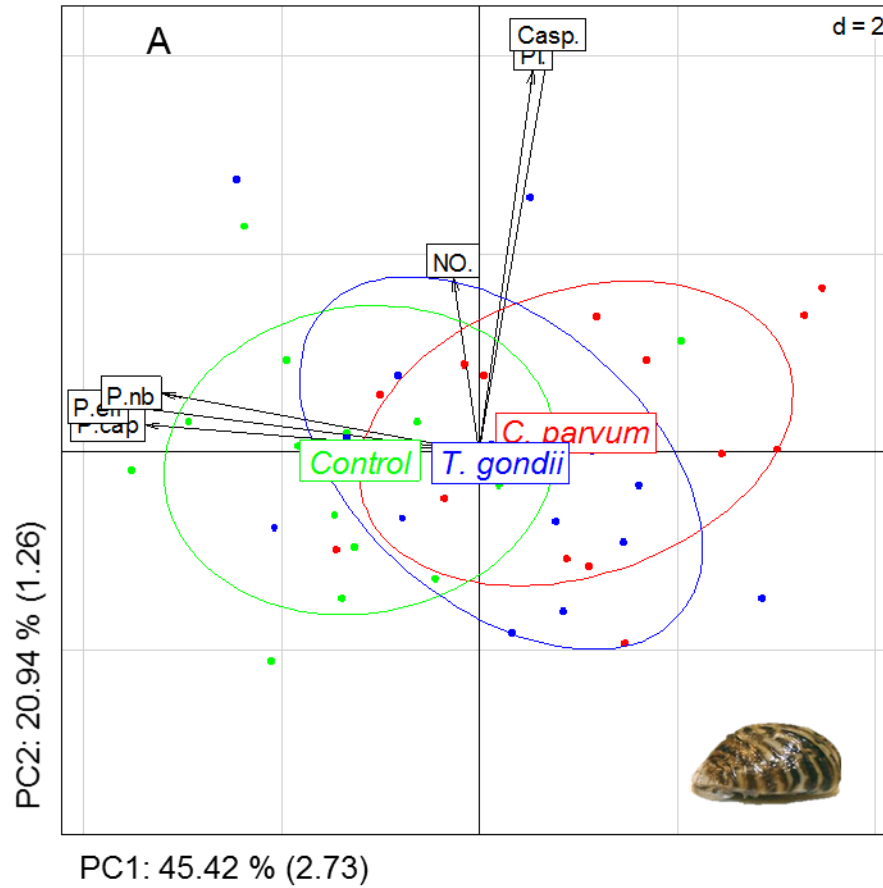
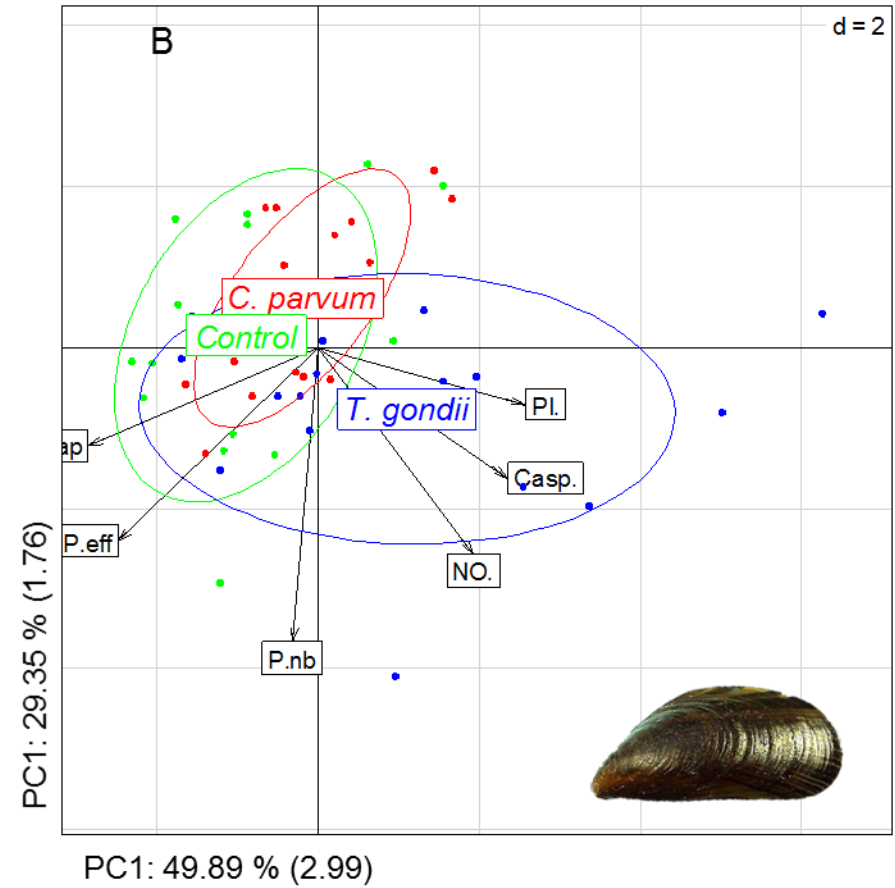


Fig. 2



Immune markers	PI+	P.cap	P.eff	P.nb	NO+	Casp+
Contribution to PC1 (%)	0.85	32.48	35.78	29.31	0.20	1.38
Contribution to PC2 (%)	42.49	0.21	0.61	1.00	8.92	46.76



Immune markers	PI+	P.cap	P.eff	P.nb	NO+	Casp+
Contribution to PC1 (%)	21.81	26.89	20.38	0.32	12.25	18.35
Contribution to PC2 (%)	1.65	4.89	19.04	43.99	21.72	8.71

Table 1. List of immune parameters analysed in this study.

Immune markers	Abbreviations	Analysis methods	Units	Ex/Em (nm)
Hemocyte mortality	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 microbeads 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

Ex, excitation wavelength; Em, emission wavelength; DEVD, DEVD tetrapeptide sequence of aspartic acid – glutamic acid – valine - aspartic acid; DAF-fm, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; NO, nitric oxide.

Table 2. Basal levels of immune markers of *Dreissena polymorpha* and *Mytilus edulis* hemocytes (mean \pm S.D.). Significant differences of immune markers between mussels (Diff) are mentioned with an asterisk with $P < 0.001$ on $n=16$. PI, propidium iodide; NO, nitric oxide; DAF-fm, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate.



<u>Basal levels</u> $n = 16$					
Immune markers	<i>Dreissena polymorpha</i>	<i>Mytilus edulis</i>	Mean \pm S.D.	Mean \pm S.D.	Diff
Hemocyte mortality (% cells labelled with PI)	11.53 \pm 2.92	4.03 \pm 2.49			*
Hemocyte apoptosis (% cells with activated caspases 3/7)	6.48 \pm 5.34	1.72 \pm 0.92			*
Phagocytic capacity (% cells that have ingested at least 1 bead)	66.97 \pm 6.61	79.78 \pm 6.18			*
Phagocytic efficiency (% cells that have ingested at least 3 beads)	42.15 \pm 8.08	58.91 \pm 9.42			*
Phagocytic avidity (mean number of ingested beads)	6.42 \pm 1.29	6.77 \pm 2.13			
NO basal production (mean DAF-fm fluorescence)	10798 \pm 7581	7775 \pm 3439			

Table 3. Immune markers of *Mytilus edulis* and *Dreissena polymorpha* hemocytes exposed to *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts at different concentrations (mean \pm S.D.). X:X ratio protozoan:hemocyte. Different letters (a, b, c) correspond to significant differences (Diff) between hemocytes exposed to different concentrations of one protozoan ($\alpha = 5\%$), with $a > b > c$ on $n=8$. Shading differentiates the effects of the two protozoa (*C. parvum* and *T. gondii*). PI, propidium iodide; NO, nitric oxide; DAF-fm, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate.

Responses to stress $n = 8$



Immune markers	<i>C. parvum</i>	<i>T. gondii</i>	<i>Dreissena polymorpha</i>		<i>Mytilus edulis</i>		
			Mean \pm S.D.	Diff	Mean \pm S.D.	Diff	
Hemocyte mortality (% cells labelled with PI)	0:1		11.37 \pm 2.47		4.10 \pm 2.85		
	1:1		13.58 \pm 3.51		4.19 \pm 1.88		
	5:1		12.75 \pm 3.32		4.25 \pm 2.61		
			0:1	11.68 \pm 3.49		3.95 \pm 2.28	b
			1:25	13.47 \pm 3.27		5.38 \pm 3.29	ab
		1;5	12.16 \pm 2.88		8.66 \pm 3.97	a	
Hemocyte apoptosis (% cells with activated caspases 3/7)	0:1		7.86 \pm 5.19		1.48 \pm 0.69		
	1:1		8.07 \pm 5.41		1.40 \pm 1.11		
	5:1		8.29 \pm 3.73		1.41 \pm 1.07		
			0:1	5.10 \pm 5.46		1.96 \pm 1.09	b
			1:25	5.39 \pm 6.01		3.73 \pm 2.57	ab
		1;5	5.35 \pm 5.44		7.01 \pm 5.33	a	
Phagocytic capacity (% cells that have ingested at least 1	0:1		65.74 \pm 8.57	a	78.32 \pm 6.81		
	1:1		59.87 \pm 8.77	ab	77.98 \pm 6.77		
	5:1		55.25 \pm 7.22	b	73.50 \pm 4.35		
		0:1	68.19 \pm 4.08	a	81.24 \pm 5.53	a	

bead)		1:25	65.96 ± 5.69	a	78.30 ± 6.72	a
		1;5	52.49 ± 4.67	b	68.56 ± 9.84	b
Phagocytic efficiency (% cells that have ingested at least 3 beads)		0:1	40.42 ± 10.64	a	53.36 ± 9.07	
		1:1	31.99 ± 9.54	ab	54.96 ± 10.39	
		5:1	27.24 ± 8.02	b	50.42 ± 6.15	
Phagocytic avidity (mean number of ingested beads)		0:1	43.89 ± 4.44	a	64.46 ± 6.13	a
		1:25	40.50 ± 7.53	a	61.48 ± 5.75	a
		1;5	26.99 ± 4.76	b	47.17 ± 10.39	b
NO basal production (mean DAF-fm fluorescence)		0:1	6.20 ± 1.55	a	5.69 ± 1.10	
		1:1	4.83 ± 1.18	ab	6.26 ± 1.30	
		5:1	4.45 ± 0.97	b	6.15 ± 1.07	
		0:1	6.65 ± 1.02	a	7.86 ± 2.41	
		1:25	5.96 ± 1.24	ab	8.20 ± 2.05	
		1;5	4.91 ± 0.84	b	6.64 ± 0.65	
		0:1	8651 ± 2422	b	6452 ± 1181	
		1:1	9551 ± 1660	ab	8799 ± 2472	
		5:1	11426 ± 2154	a	10211 ± 3843	
		0:1	12945 ± 10333		9098 ± 4467	b
		1:25	10332 ± 2648		11365 ± 4533	ab
		1;5	12155 ± 3376		15857 ± 4977	a

Without stress

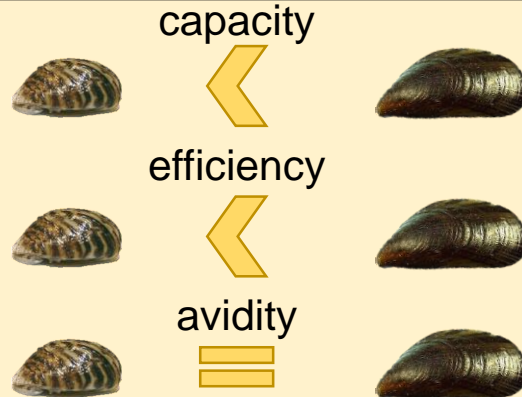
➤ Hemocyte mortality



➤ Hemocyte apoptosis



➤ Phagocytosis

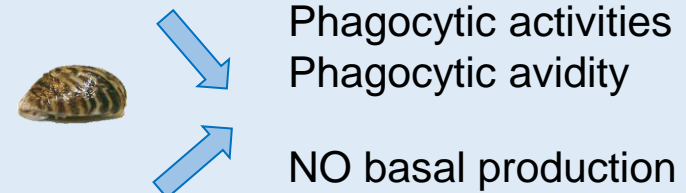
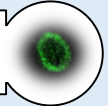


➤ Nitric oxide production



Exposed to protozoa

➤ to *C. parvum*



➤ to *T. gondii*

