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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Biological models

2.1.1. Zebra and blue mussels

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

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

2.1.2. Hemolymph collection

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

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

2.2. Exposures to biological stress and assessment of immune parameters

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

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

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

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

2.3. Statistical analyses

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

3. Results and discussion

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

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

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

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

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

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

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

3.2. Effects of human protozoa on immune parameters of mussels

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

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

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

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

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

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

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

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

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

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

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

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

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

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Figure legends

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

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

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

Fig. 1

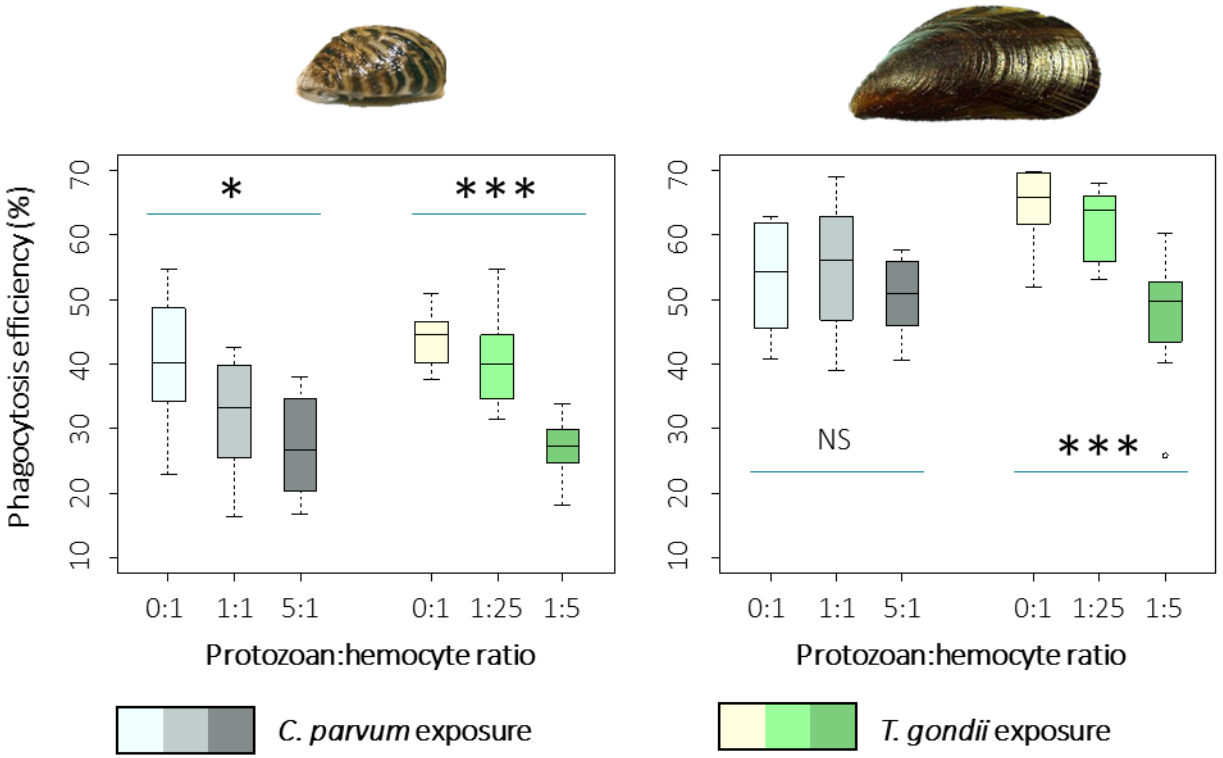
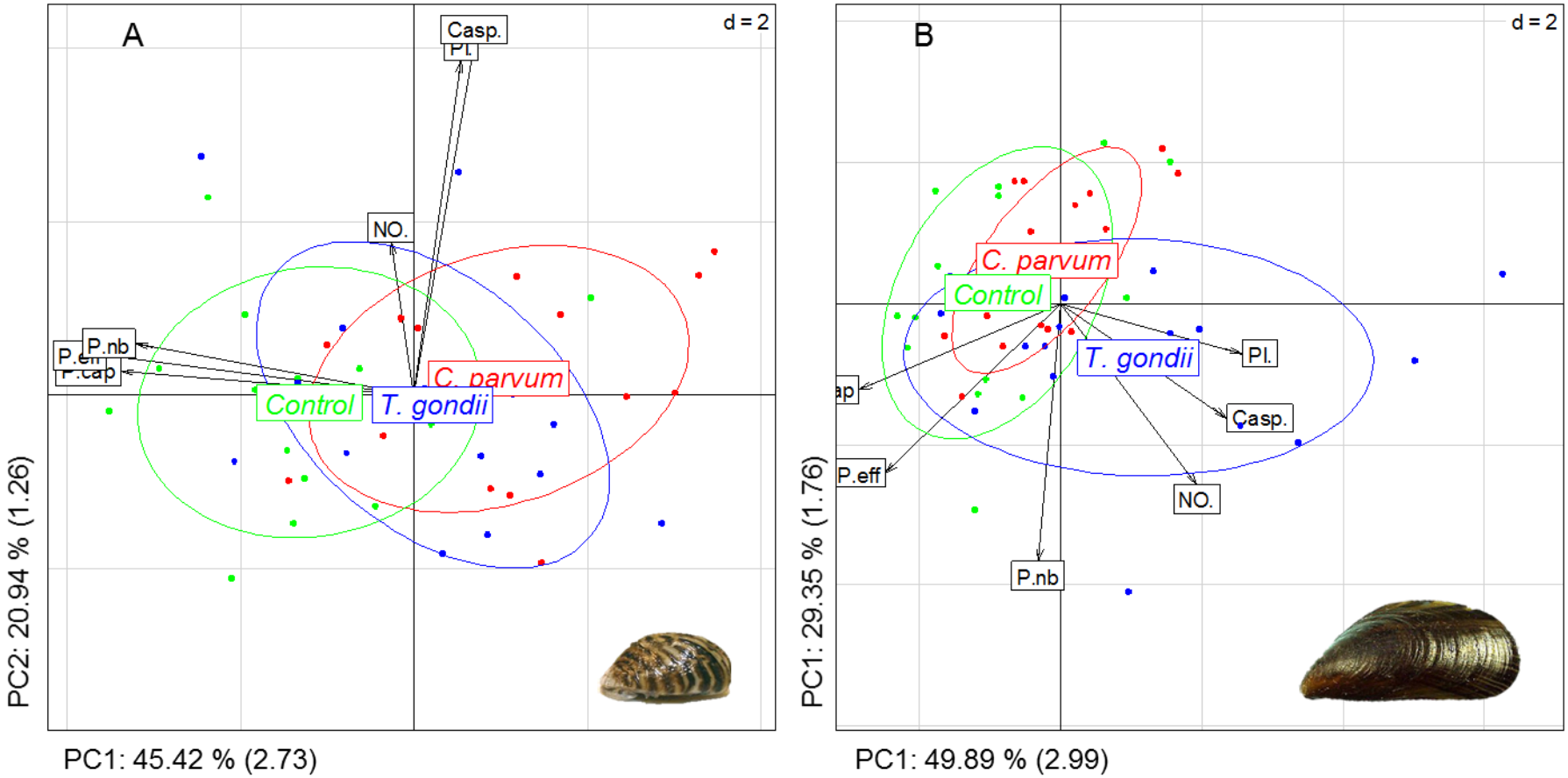

Phagocytosis efficiency (%)
10
20
30
40
50
60
70
*
***
0:1
1:1
5:1
0:1
1:25
1:5
Protozoan:hemocyte ratio
C. parvum exposure
NS
***
0:1
1:1
5:1
0:1
1:25
1:5
Protozoan:hemocyte ratio
T. gondii exposure

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 ± 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.

Basal levels $n = 16$

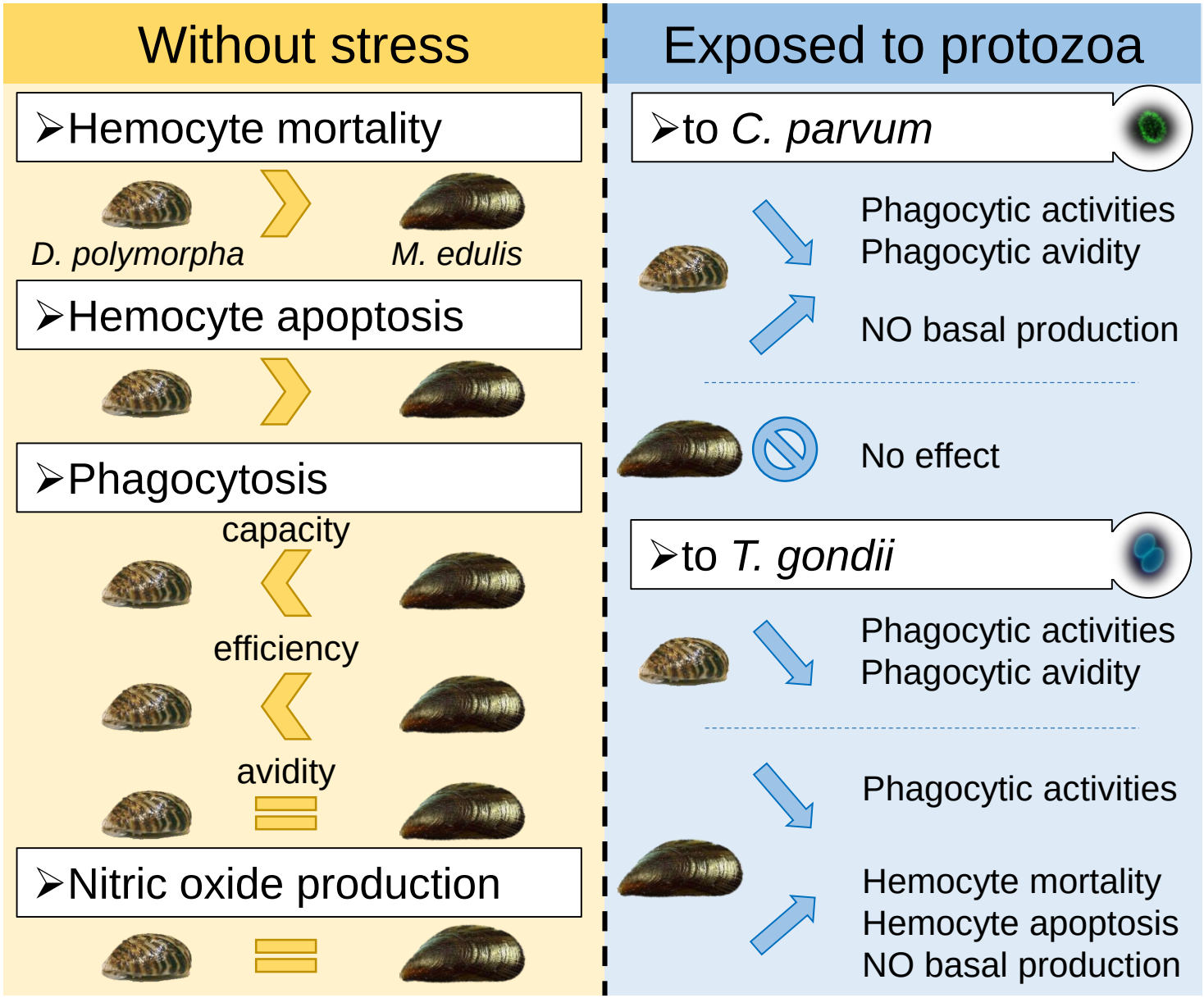
Immune markers	***Dreissena polymorpha*** Mean ± S.D.	***Mytilus edulis*** Mean ± S.D.	Diff
Hemocyte mortality (% cells labelled with PI)	11.53 ± 2.92	4.03 ± 2.49	*
Hemocyte apoptosis (% cells with activated caspases 3/7)	6.48 ± 5.34	1.72 ± 0.92	*
Phagocytic capacity (% cells that have ingested at least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

Table 3. Immune markers of *Mytilus edulis* and *Dreissena polymorpha* hemocytes exposed to *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts at different concentrations (mean ± 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 (α = 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	*C. parvum*	*T. gondii*	*Dreissena polymorpha* Mean ± S.D.	Diff	*Mytilus edulis* Mean ± S.D.	Diff
Hemocyte mortality (% cells labelled with PI)	0:1		11.37 ± 2.47		4.10 ± 2.85	
	1:1		13.58 ± 3.51		4.19 ± 1.88	
	5:1		12.75 ± 3.32		4.25 ± 2.61	
		0:1	11.68 ± 3.49		3.95 ± 2.28	b
		1:25	13.47 ± 3.27		5.38 ± 3.29	ab
		1;5	12.16 ± 2.88		8.66 ± 3.97	a
Hemocyte apoptosis (% cells with activated caspases 3/7)	0:1		7.86 ± 5.19		1.48 ± 0.69	
	1:1		8.07 ± 5.41		1.40 ± 1.11	
	5:1		8.29 ± 3.73		1.41 ± 1.07	
		0:1	5.10 ± 5.46		1.96 ± 1.09	b
		1:25	5.39 ± 6.01		3.73 ± 2.57	ab
		1;5	5.35 ± 5.44		7.01 ± 5.33	a
Phagocytic capacity (% cells that have ingested at least 1	0:1		65.74 ± 8.57	a	78.32 ± 6.81	
	1:1		59.87 ± 8.77	ab	77.98 ± 6.77	
	5:1		55.25 ± 7.22	b	73.50 ± 4.35	
		0:1	68.19 ± 4.08	a	81.24 ± 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
	0:1		40.42 ± 10.64	a	53.36 ± 9.07	
	1:1		31.99 ± 9.54	ab	54.96 ± 10.39	
Phagocytic efficiency	5:1		27.24 ± 8.02	b	50.42 ± 6.15	
(% cells that have ingested at least 3 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
	0:1		6.20 ± 1.55	a	5.69 ± 1.10	
	1:1		4.83 ± 1.18	ab	6.26 ± 1.30	
Phagocytic avidity	5:1		4.45 ± 0.97	b	6.15 ± 1.07	
(mean number of ingested beads)		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	
NO basal production	5:1		11426 ± 2154	a	10211 ± 3843	
(mean DAF-fm fluorescence)		0:1	12945 ± 10333		9098 ± 4467	b
		1:25	10332 ± 2648		11365 ± 4533	ab
		1;5	12155 ± 3376		15857 ± 4977	a


Without stress
Exposed to protozoa
➢Hemocyte mortality
D. polymorpha
M. edulis
➢Hemocyte apoptosis
➢Phagocytosis
capacity
efficiency
avidity
➢Nitric oxide production
➢to C. parvum
Phagocytic activities
Phagocytic avidity
NO basal production
No effect
➢to T. gondii
Phagocytic activities
Phagocytic avidity
Phagocytic activities
Hemocyte mortality
Hemocyte apoptosis
NO basal production