Fish & Shellfish Immunology
September 2016, Volume 56, Pages 322-329
http://dx.doi.org/10.1016/j.fsi.2016.07.021
http://archimer.ifremer.fr/doc/00346/45692/

Flat oyster follows the apoptosis pathway to defend against the protozoan parasite *Bonamia ostreae*

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Abstract :

The *in vitro* model *Ostrea edulis* hemocyte - *Bonamia ostreae* is interesting to investigate host-parasite interactions at the cellular level. Indeed, this unicellular parasite infects the flat oyster *Ostrea edulis* and multiplies within hemocytes, the central effectors of oyster defenses. Apoptosis is a mechanism used by many organisms to eliminate infected cells. In order to study the potential involvement of this mechanism in the oyster response to *B. ostreae*, *in vitro* experiments were carried out by exposing hemocytes from the naturally susceptible oyster *O. edulis* and a resistant oyster species *Crassostrea gigas* to live and heat-inactivated parasites. Hemocyte apoptotic response was measured using a combination of flow cytometry and microscopy analyses. Whatever the host species was, the parasite was engulfed in hemocytes and induced an increase of apoptotic parameters including intracytoplasmic calcium concentration, mitochondrial membrane potential or phosphatidyl-serine externalization as well as ultrastructural modifications. However, the parasite appears more able to infect flat oyster than cupped oyster hemocytes and the apoptotic response was more important against live than dead parasites in the natural host than in *C. gigas*. Our results suggest that *O. edulis* specifically responds to *B. ostreae* by inducing apoptosis of hemocytes.

Highlights

► Apoptosis in flat and cupped oysters was investigated in the context of *in vitro* infections with the protozoan parasite *Bonamia ostreae*. ► Contact with live and inactivated *Bonamia ostreae* induces modifications of tested apoptosis parameters in both *Ostrea edulis* and *Crassostea gigas* oyster species. ► The apoptotic response in the flat oyster *Ostrea edulis* seems to be more specific to *Bonamia ostreae* than in the cupped oyster *Crassostrea gigas*. ► Apoptosis: an important mechanism developed by *Ostrea edulis* against bonamiosis.

Keywords : Apoptosis, Host-parasite interactions, Parasite, Oyster, *Bonamia ostreae*, *Ostrea edulis*

1. Introduction

The flat oyster *Ostrea edulis* is the European endemic oyster species. In the 70's the production was dramatically reduced in relation with a combination of factors including the disease caused by the unicellular protozoan parasite *Bonamia ostreae*. Once the parasite is in an oyster population there is no way to eradicate it and to treat oysters. Reviving the production of *O. edulis* in an infected zone notably relies on favouring resistant or tolerant oysters and requires a better understanding of host parasite interactions, in particular mechanisms involved in the response against *B. ostreae*. Oyster defenses not only rely on the anatomical and chemical barriers including the shell and palleal organs but also mucus and cellular and soluble components of hemolymph like hemocytes, lysosomal enzymes and lectins [1].

In *O. edulis*/*B. ostreae* host/parasite model, hemocytes play a key role by being central effectors of oyster defenses and target cell of the parasite. Despite the lack of parasite and hemocyte cultures, an *in vitro* experimental infection model has been developed by incubating hemocytes with parasites purified from highly infected oysters [2]. This experimental model allowed demonstrating that parasites are internalized after 30 min of contact with hemocytes [3] and are able to persist and multiply within hemocytes after 2h of contact [4]. Hemocytes internalyze parasites by endocytosis but *B.ostreae* seem to actively contribute to its own internalization [3]. It has recently been shown that a heat shock protein 90 (HSP90) is partly involved in parasite internalization [5]. Once internalized, *B.ostreae* induces some modifications of hemocyte activities including decrease of reactive oxygen species (ROS) production and esterase activities [4,6]. Previous studies suggested a potential involvement of apoptosis in the response of the oyster against the parasite. Genes involved in the apoptosis pathway including Fas ligand, an inhibitor of apoptosis (IAP), Tumor necrosis factor (TNF) and apoptosis inducer factor (AIF) were found modulated in the context of experimental or field infections [7,8].

Apoptosis is a highly orchestrated process developed in many organisms and plays a role in various biological processes like embryogenesis, homeostasis and particularly in defense against stress factors including pathogens by eliminating infected cells [9,10]. This process induces various morphological changes like chromatin condensation, membrane blebbing and in the last stages cells fragmentation into apoptotic bodies [10]. There are two main apoptotic pathways: the extrinsic pathway initiated by activation of membrane receptor like TNF receptor and Fas receptor and the intrinsic or mitochondrial pathway activated by cellular stress [9,11].

In mollusc, apoptosis seems to be an important mechanism of defense against stress factors including environmental disturbances or pathogens [1,12,13]. *In vitro* studies have shown that oyster hemocyte apoptosis is increased by UV exposure [14] or by exposure to pesticides and heavy metals [15,16]. This mechanism leads to the death of the exposed cells. Some pathogens are able to modulate this phenomenon to favour their survivor and multiplication within hemocytes [17–19]. For example, a virulent strain of the protozoan parasite *Perkinsus marinus* was able to inhibit apoptosis of

Crassostrea virginica hemocytes after few hours of *in vitro* contact contrary to a low virulent strain [20]. A differential apoptotic response was also reported between *C. virginica* and *Crassotrea gigas*, two species presenting different levels of susceptibility to *P. marinus*. After 3 days, percentage of apoptotic hemocytes was more important in Pacific oyster suggesting a possible activation of apoptosis as defense mechanism more important in the more resistant species [21].

As previously mentioned, genes involved in apoptosis appear to be modulated in *O. edulis* during an infection with *B. ostreae*. Considering these results, our objectives were to (i) describe more precisely the apoptotic hemocyte reaction after a contact with the parasite *B.ostreae* and (ii) compare the apoptotic process between hemocytes in contact with alive and heat inactivated parasites and between hemocytes from *O. edulis* and *C. gigas*, a susceptible and resistant oyster species respectively.

The combination of flow cytometry and microscopic tools recently validated to investigate apoptosis in flat oyster hemocytes [14] were used to carry out the present study.

2. Material and methods

2.1. Oysters

Adult flat oysters (> two-year-old) *O. edulis* were collected from Quiberon bay (Brittany, France) in October 2013 and were acclimatized in Ifremer's facilities (La Tremblade, Charente maritime, France) during two months. Pacific oysters *C. gigas* from bi-parental family produced at Ifremer's facilities in June 2013 were used for this experimentation. Oysters from both species were maintained in raceways (12000 L) supplied with a constant flow of seawater enriched in phytoplankton (*Skeletonema costatum*, *Isochrisis galbana* and *Tetraselmis succica*).

2.2. Hemolymph collection

Hemolymph was withdrawn from the hemolymphatic sinus of the adductor muscle of oysters with a 1 mL syringe and a needle of 0.60 x 25 mm. Hemolymphs were kept on ice to avoid cellular aggregation

and were filtered at 75 µm to remove debris and cell aggregates. Hemocytes were then counted using a hemocytometer and concentration adjusted at $5x10^5$ cells.mL^{-1} by adding 0.22 µm filtered sea water (FSW).

2.3. Parasites

B. ostreae was purified according to the protocol developed by [22]. Heavily infected oysters were selected by examination of gill tissue imprints using light microscopy. After homogenization of all organs except the adductor muscle, parasites were concentrated and purified by differential centrifugation on sucrose gradients. Finally, *B. ostreae* were suspended in FSW, counted using a hemocytometer and kept at 4°C. Viability of parasite was tested with propidium iodide (PI) by flow cytometry according to Arzul et al. (2009). Parasite species was confirmed by RFLP PCR according to the following Standard Operating Procedure : http://www.eurl-mollusc.eu/content/download/15841/236614/file/BonamiaPCR-RFLP_SOP.pdf. Only *B. ostreae* was detected in parasite suspensions.

2.4. In vitro contact experiments

Hemocytes (5.10^5 cells. mL^{-1}) of *O. edulis* and *C. gigas* were incubated with live and heat inactivated parasites (100°C for 15 min) at the ratio of 10:1 parasites per hemocyte at 15 °C. The control consisted of non-treated hemocytes suspended in FSW (5.10^5 cells.mL^{-1}). Hemocytes were analyzed after 1, 2 and 4 hours of incubation with parasites. Each condition was tested in duplicate and the whole experiment was carried out three times for flat oysters and twice for Pacific oysters.

2.5. Light microscopy

For each sampling time and experimental condition, 100 µl of cell suspension were centrifuged for 1 min at 28 x g and 4°C, stained with Hemacolor® (Merk) and examined using light microscopy.

2.6. Flow cytometry analyses

Hemocyte intracytoplasmic calcium concentration, mitochondrial membrane potential (ΔΨm) and phosphatidyl-serine externalisation were monitored by flow cytometry using an EPICS XL 4 (Beckman coulter) according to Gervais et al. (2015). Six replicates were tested for each condition.

2.7. DNA fragmentation (TUNEL)

DNA fragmentation was evaluated on cytocentrifuged hemocyte suspensions using the *In situ* Cell Death Detection Kit, POD (Roche) following Gervais et al. (2015). Six slides were observed for each condition.

2.8. Transmission electron microscopy (TEM)

Hemocyte suspensions (1.10^6 cells) were centrifuged at 500 x g for 8 min at 4°C and supernatant was discarded. Samples were fixed in 3% glutaraldehyde solution for 1 day at 4 °C and processed as described in Gervais et al. (2015). Two conditions were observed for both oyster species: non treated cells at 4 h and cells in contact with *B. ostreae* after 4 h.

2.9. Statistical analyses

A non-parametric Wilcoxon test was used to estimate the effect of *B. ostreae*, oyster species and time on the apoptotic parameters. To compare two groups, pairwise Wilcoxon test was performed. A discriminant analysis was applied on flow cytometry parameters and percentage of “infected” cells to identify potential impact of tested conditions. To confirm separation between all the groups Wilk’s lambda test was performed. Estimation of the correlation between each tested parameter was done with Spearman test. Significance was set at $p<0.05$ (*) for all the tests. Statistic test was performed using JMP 10.0.0 software (SAS institute Inc.).

3. Results

3.1. Parasite viability

Status of purified parasites was checked by flow cytometry prior to each experiment. No more than 24% of mortality was observed in suspensions of live parasites with an average of 17.16% ± 4.31. After heat inactivation, mean parasite mortality reached 80.6% ± 19.02.

3.2. Detection of parasites in hemocytes

Whatever the oyster species and the status of *B. ostreae* (alive or inactivated) were, more than 10% of hemocytes have engulfed parasite as soon as 1 h of contact between hemocytes and *B. ostreae* (Fig. 1 A). The percentage of infected hemocytes was similar between the two species except at 1 h of contact, percentage was significantly lower ($p<0.05$) in *C. gigas* in contact with live parasite in comparison with *O. edulis*. The percentage of cells with live parasites was significantly higher than with dead parasites after 1 h of contact for *O. edulis* and after 2 and 4 h of contact for both oyster species($p<0.05$). An increase of cells with internalized parasites was also observed between 1 and 2 h of contact for both oyster species ($p<0.05$).

The mean parasite number between *O. edulis* and *C. gigas* was significantly different at 1 h of contact ($p<0.05$) with live and inactivated parasites but was only different for live parasites at 2 h of contact ($p<0.05$) (Fig. 1 B). Number of *B. ostreae* in "infected" hemocytes was higher when parasites were alive than dead except at 1 h for the flat oyster and only after 4 h for the Pacific oyster ($p<0.05$). Mean number of parasites per "infected" hemocyte did not significantly evolve during the time of the experiment except for the both species after 4 h of contact with heat inactivated parasites.

3.3. Impact of Bonamia ostreae on early apoptotic hemocyte parameters

Early apoptotic response of hemocytes was evaluated by measuring cytoplasmic calcium concentration and $\Delta\Psi m$.

The percentage of cells positive for cytoplasmic calcium was the same between controls and cells in contact with inactivated parasites whatever the oyster species was. Hemocytes in contact with live parasite showed less positive cells than with inactivated parasites as soon as 1 h post contact for both

158 oyster species but more significantly for *O. edulis* ($p<0.05$) (Fig. 2). This percentage did not evolve
159 during the time of the experiment.

160 Contact with inactivated and live parasites induced an increase of cells with low $\Delta\Psi m$ as soon as 1 h
161 post contact and during all the experiment (Fig. 3). This increase appeared higher when parasite was
162 alive than inactivated more particularly for *O. edulis* ($p<0.05$).

163 *3.4. Impact of Bonamia ostreae on hemocyte plasma membrane integrity*

164 Hemocytes membrane integrity was evaluated by measuring phosphatidyl serine externalization. For
165 the flat oyster, no significant difference was observed between the control and hemocytes in contact
166 with heat inactivated parasites (Fig. 4) whereas live parasites induced a significant increase of labeled
167 cells. In contrast, a significant increase of positive *C. gigas* hemocytes was observed in presence of
168 live and inactivated parasites in flat oyster. No difference was observed during the time of the
169 experiment.

170 *3.5. Modulation of DNA integrity of hemocytes*

171 Whatever the tested condition was, no more than 14% of cells were labeled (Fig. 5). The only
172 significant difference was observed for hemocytes from flat oysters in contact with live parasites.

173 *3.6. Discriminant analysis*

174 A discriminant analysis was carried out to determine if apoptotic parameters tested in flow cytometry
175 and TUNEL allowed discriminating tested conditions. To confirm group discrimination, a Wilk's
176 lambda test was carried out and showed that groups were clustered by species and tested experimental
177 condition ($p<0.001$) but not by the time. Three groups could be identified: (i) a first group included
178 control of both oyster species; (ii) a second group included hemocytes in contact with inactivated
179 parasites for both oyster species and in contact with live parasites for *C. gigas* only;(iii) a last group
180 included only hemocytes of *O. edulis* after contact with live parasites (Fig. 6). Parameters
181 "cytoplasmic calcium", "mitochondrial membrane potential" and "externalization of phosphatidyl

serine" appeared correlated whereas "DNA fragmentation" was not correlated with the other tested parameters.

3.7. Correlation test

Potential correlation between parameters was tested using a Spearman test. A positive strong correlation was observed between the percentage of "infected" hemocytes and ΔΨm/externalization of phosphatidyl serine and between ΔΨm and the externalization of phosphatidyl serine (Fig. 7). A negative strong correlation was found between the intracytoplasmic calcium concentration and the percentage of "infected" hemocytes, ΔΨm and the externalization of phosphatidyl serine. In contrast, DNA fragmentation (measured using TUNEL) was not correlated with the other tested parameters.

3.8. Morphological changes

Similarly to results obtained by flow cytometry, less apoptotic cells were observed by transmission electron microscopy in the controls for both oyster species (Table1, Fig. 8A, D). Parasites were noticed within hemocytes after 4 h of contact whatever the oyster species was (Fig. 8C, F). Apoptotic hemocytes were observed among the cells which have internalized parasites but also among the "non-infected" ones for both oyster species (Fig. 8B-C, E-F). Apoptotic modifications included chromatin condensation (Fig. 8B-C, E-F), detachment of the nuclear membrane (Fig. 8B-C) and vacuolization of the cytoplasm (Fig. 8B).

4. Discussion

Apoptosis is a cellular mechanism that leads to the cell death [9,10]. This pathway is universal among metazoan organisms and related pathways have also been described in unicellular organisms like *Trypanosoma spp*., *Plasmodium spp*., *Toxoplasma spp*. and *Leishmania spp*. [24–26]. It is a mechanism that allows eliminating damaged cells or cells infected with pathogens. However, some pathogens are able to modulate apoptosis of cells involved in immunity to evade host immune defense

206 or to inhibit apoptosis of the cells they infect in order to survive and multiply within the target cells
207 [18,27].

208 Few studies have been carried out to investigate apoptosis in molluscs in response to diseases. Most of
209 these works focused on the parasite *P. marinus* in *C. virginica* [20,21,28]. However, the specificity of
210 this response has never been questioned.

211 Previous studies have suggested the potential involvement of apoptosis in the response of the flat
212 oyster *O. edulis* against an infection with the protozoan parasite *B. ostreae* [8]. This hypothesis was
213 supported by a modulation of the expression of oyster genes involved in the apoptosis pathway
214 including IAP and Fas ligand in presence of the parasite [8]. In order to confirm this molecular
215 evidence, we have investigated the apoptotic response of hemocytes exposed to parasites *in vitro* by
216 combining microscopic and cellular tools previously developed [14]. Additionally, the specificity of
217 this response has been tested by comparing the response of the susceptible species *O. edulis* with the
218 response of a species considered as resistant, *C. gigas* [29,30].
219 *In vitro* experimental infection showed that as soon as 1 h of contact some heat inactivated and live
220 parasites were internalized by hemocytes of both oyster species. After 2 hours, “infected” hemocytes
221 were more abundant when parasites were alive than heat inactivated. Generally, more “infected”
222 hemocytes were observed when cells were in contact with live parasites than heat inactivated parasites
223 for *O. edulis* than *C. gigas*. These results are in agreement with previous studies showing that *B.*
224 *ostreae* was involved in its own internalization. Indeed, *B. ostreae* treatment consisting in blocking
225 actin and HSP90 drastically reduced the percentage of “infected” cells [3,5,6].

226 In flat oyster hemocytes, contact with live parasites increased apoptosis as revealed by a decrease of
227 cytoplasmic calcium and mitochondrial membrane potential and an increase of phosphatidyl serine
228 externalization, DNA fragmentation and ultrastructure alterations. These modifications appeared
229 significantly higher when parasites were alive compared to heat inactivated parasites which suggests
230 that live *B. ostreae* actively induces hemocyte apoptosis. Studies on *P. marinus* showed that
231 inactivation of the parasite did not modify early apoptotic response of hemocytes in *C. virginica* [20].

TEM examination showed that apoptosis in hemocytes in contact with parasites occurred in "infected" cells but also in "non-infected" hemocytes. This response can be triggered by the parasite directly or by infected cells. Apoptosis might allow the oysters to decrease the number of available host cells and thus the capacity of the parasite to develop and multiply. If apoptosis can be considered as beneficial for the oyster it can also contribute to affect the oyster itself by depleting the population of hemocytes. This early specific activation of apoptosis suggests that there is a specific interaction between hemocytes of *O. edulis* and *B. ostreae*. This interaction could rely on the recognition and binding of *B. ostreae* with an hemocyte receptor activating the apoptosis pathway. A previous study has shown that the galectin, Oe-Gal, can interact with *B. ostreae* and plays a role in the internalization of the parasite (Prado-Alvarez, Unpublished). Galectins can modulate cell apoptosis [31–34]. During infection, they can inhibit parasite replication and commit cells to death by apoptosis [33–35].

Basal apoptotic rate in control condition was the same in both oyster species. Both alive and heat treated parasites induced an increase of hemocytes apoptosis in flat oyster and cupper oysters. However, difference could be noticed between both oyster species: for example, percentage of cells with intracytoplasmic calcium decreased more significantly in presence of live parasite in *O. edulis* than in *C. gigas*. These results suggest that the parasite is more able to actively induce its own internalization by modulating cytoskeleton in hemocytes from *O. edulis* than in *C. gigas*. Similarly, pathogens like *Trypanosoma cruzi* are known to favour their entry in the host cells by modulating host cytoskeleton through a change of intracellular calcium [36]. These results are also supported by the higher percentage of infected hemocytes for *O. edulis* compared to *C. gigas*.

Hemocyte $\Delta\Psi m$ was enhanced by the contact with parasites whatever the oyster species and the parasite status were. However, this increase was significantly higher in *O. edulis* when the parasite was alive. Moreover, hemocyte membrane integrity appeared only altered in flat oyster when *B. ostreae* was alive whereas in *C. gigas* it was modified similarly in presence of live and dead parasites. Finally, DNA fragmentation was significantly increased only in flat oyster hemocytes after contact with live *B. ostreae*. Thus, contrary to results observed in *O. edulis*, less difference was found in *C. gigas* between hemocytes in contact with alive and heat-treated parasites suggesting that defense

strategy against the parasite is different in both oyster species. Incidentally, *C. gigas* has never been detected infected with *B. ostreae* implying that confrontation between hemocytes and *B. ostreae* should rarely occur.

Combining different cellular tools allowed us discriminating between tested conditions. Correlations observed between cytoplasmic calcium concentration, mitochondrial membrane potential and externalization of phosphatidyl serine suggest that it not necessary to measure all these parameters to monitor apoptosis in hemocyte in response to *B. ostreae*. However, using complementary approaches including cytometry and microscopy allows describing more widely early modifications induced by an *in vitro* infection with *B. ostreae*. For the first time we showed that *B. ostreae in vitro* infection induces apoptosis not only in hemocytes of flat oyster *O. edulis* but also of *C. gigas*. However, *B. ostreae* appears more efficient to infect *O. edulis* than *C. gigas* hemocytes and the flat oyster seems to respond more specifically to live parasites by increasing hemocyte apoptosis than *C. gigas*. Although hemocytes of *C. gigas* were less infected, percentages of apoptotic hemocytes were higher than in *O. edulis*. This result might partly explain the resistance of *C. gigas* against *B. ostreae*.

Our experiments were probably not long enough to observe a potential modulation of this response by the parasite itself. Indeed, previous studies carried out *in vitro* showed that hemocyte apoptosis induced by different *P. marinus* strains was inhibited after 5 hour of contact [20]. Complementary *in vivo* infection would also be helpful to draw an integrative picture of this mechanism in response to an infection in molluscs.

Acknowledgements

The authors wish to thank all members of the staff of the Ifremer hatchery in La Tremblade for their technical assistance for the maintenance of oysters in Ifremer La Tremblade, France. We gratefully acknowledge L. Degremont for his involvement in the production of the Pacific oyster family used in the present study. We thank A. Langlade for supplying flat oysters from Quiberon. This work was supported by Ifremer and the Région of Poitou Charentes, France.

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Figure 1: Percentage of hemocytes with internalized live or inactivated *Bonamia ostreae* in *Ostrea edulis* and *Crassostrea gigas* during the time (A). Number of parasites per hemocyte of *O. edulis* and *C. gigas* (B). Boi: *B. ostreae* inactivated, Bo: *B. ostreae*. Results represent the mean±SD. a: significant difference with inactivated parasites ($p<0.05$), b: significant difference with flat oysters ($p<0.05$) c: significant difference with 1h ($p<0.05$), d: significant difference with 2h ($p<0.05$).

Figure 2: Modulation of cytoplasmic calcium concentration in hemocytes of *Ostrea edulis* and *Crassostrea gigas* following *Bonamia ostreae* exposure. Percentage of labeled hemocytes with cytoplasmic calcium concentration depending on the oyster species and the exposure to *B. ostreae* during the time. Results represent the mean±SD of three experiments. control: non exposed, Boi: *B. ostreae* inactivated, Bo: *B. ostreae*. a: significant difference with control ($p<0.05$), b: significant difference with *B. ostreae* inactivated ($p<0.05$), c: significant difference with *O. edulis* ($p<0.05$).

Figure 3: Modulation of mitochondrial membrane potential (ΔΨm) in hemocytes of *Ostrea edulis* and *Crassostrea gigas* following *Bonamia ostreae* exposure. Percentage of labeled hemocytes with low ΔΨm in function of the oyster species and exposure or not to *B. ostreae* during the time. Results represent the mean±SD of three experiments. control: non exposed, Boi: *B. ostreae* inactivated, Bo: *B. ostreae*. a: significant difference with control ($p<0.05$), b: significant difference with *B. ostreae* inactivated ($p<0.05$), c: significant difference with *O. edulis* ($p<0.05$).

Figure 4: Modulation of plasma membrane integrity in hemocytes of *Ostrea edulis* and *Crassostrea gigas* following *Bonamia ostreae* exposure. Percentage of AnnexinV positive and PI negative hemocytes depending on the oyster species and the exposure to *B. ostreae* during the time. Results represent the mean±SD of three experiments. control: non exposed, Boi: *B. ostreae* inactivated, Bo: live *B. ostreae*. a: significant difference with control ($p<0.05$), b: significant difference with *B. ostreae* inactivated ($p<0.05$), c: significant difference with *O. edulis* ($p<0.05$).

Figure 5: Modulation of DNA integrity of *Ostrea edulis* and *Crassostrea gigas* following *Bonamia ostreae* exposure. Percentage of TUNEL positive hemocytes in function of the oyster species and exposure or not to *B. ostreae* during the time. Results represent the mean±SD of three experiments. control: non exposed, Boi: *B. ostreae* inactivated, Bo: *B. ostreae*. a: significant difference with *B. ostreae* inactivated ($p<0.05$), b: significant difference with 1 h ($p<0.05$).

Figure 6: Discriminant analysis of flow cytometry parameters depending on tested conditions. Oe: *Ostrea edulis* represented in shade of blue, Cg: *Crassostrea gigas* represented in shade of grey. Boi: hemocyte in contact of inactivated *Bonamia ostreae* represented by small dot line, Bo: hemocyte in contact of live *B. ostreae* represented by big dot line. 1, 2 and 4: time of the experiment post contact with B. ostreae, the darkest shade represent lower time and the lightest shade represent the longest contact time.

Figure 7: Correlation between tested parameters. Negative and positive correlations are shown in blue and red respectively.

Figure 8: Morphological changes observed after 4h of contact with *Bonamia ostreae* by transmission electron microscopy. *Ostrea edulis*: (A) control cell, (B) apoptotic hemocyte without *B. ostreae* internalization, (C) apoptotic hemocyte with B. ostreae internalization. *Crassostrea gigas*: (D) control cell, (E) apoptotic hemocyte without internalization of *B. ostreae*, (F) apoptotic hemocyte with *B. ostreae* internalization. Arrowheads*: B. ostreae*.

427 **Table 1.** Percentages of different cells status among hemocytes of *Ostrea edulis* or *Crassostrea gigas*
428 oysters after 4 h of contact or not with *Bonamia ostreae*.

		% of normal cells	% of apoptotic cells	% of secondary necrotic cells	% of necrotic cells
O. edulis	Control (n=59)	61	27.1	3.4	8.1
	B. ostreae (n=55)	38.18	50.91	3.64	7.27
C. gigas	Control (n=52)	88.46	11.54	0	0
	B. ostreae (n=45)	35.56	57.78	0	6.67

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