**Fish & Shellfish Immunology**
June 2009, Volume 26, Issue 6, Pages 836-842
http://dx.doi.org/10.1016/j.fsi.2009.03.018




# Infection with the protozoan parasite Bonamia ostreae modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*

Benjamin Morga[1], Isabelle Arzul[1,] *, Bruno Chollet[1] and Tristan Renault[1]

[1] Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER); Laboratoire de Génétique et Pathologie (LGP); La Tremblade, France

*: Corresponding author : I. Arzul, Phone: 33 5 46 76 26 10, Fax: 33 5 46 76 26 11, email address : iarzul@ifremer.fr

**Abstract:**

*Bonamia ostreae* is an intracellular protozoan parasite, infecting haemocytes of the European flat oyster *Ostrea edulis*. Oyster defence mechanisms mainly rely on haemocytes. In the present study in vitro interactions between parasites and flat oyster haemocytes were investigated using flow cytometry and light microscopy.
Haemocyte parameters including: non specific esterase activity, reactive oxygen species (ROS) production and phagocytosis were monitored using flow cytometry after 2 hour cell incubation with live and dead *B. ostreae*. Two ratios of parasites per haemocyte were tested (5:1 and 10:1), haemocytes alone were used as controls and the experiment was carried out three times. Flow cytometry revealed a decrease of non specific esterase activities and ROS production by haemocytes after incubation with live parasites, while there was little difference in phagocytosis activity when compared with controls. Similarly, dead parasites induced a decrease in haemocyte activities but to a lesser extent compared to live parasites. These results suggest that *B. ostreae* actively contributes to the modification of haemocyte activities in order to ensure its own intracellular survival.

**Keywords:** *Bonamia ostreae, Ostrea edulis*, Haemocytes, Flow cytometry, Esterase, Reactive oxygen species (ROS), Phagocytosis.

# 1. Introduction

The European flat oyster *Ostrea edulis* occurs from Norway to the Bay of Agadir in Morocco and in the Mediterranean and Black Seas. In the late 1960's decline in flat oyster production was attributed to the protozoan parasite *Marteilia refringens* [1, 2, 3]. Since 1979, flat oyster production has further declined in France and Europe due to another disease caused by the protozoan parasite, *Bonamia ostreae* [4, 5, 6]. Both parasite diseases contributed to drastically reduce the French flat oyster production from 20 000 t per year in the 1970s to 1 000 t (data available in the fishstat database, http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp). *B.ostreae* was also reported in cultured and wild populations from the Netherlands [7, 8], Denmark [9], Spain [10], United Kindom [11] and Ireland [12].

The natural host of the protozoan parasite *Bonamia ostreae* is the European flat oyster *Ostrea edulis*. Although the parasite can be observed in juveniles [13] and adults, mortality usually occurs in oysters 2 year old and older [14, 15, 16, 17]. The parasite is most often observed inside the haemocytes [4, 5], but it can also be detected free in connective tissues of the digestive gland and gills [18]. Multiplication of the parasite is associated with haemocyte disruption and huge haemocyte infiltration in the digestive gland, mantle and gills [14, 19]. Phylogeny studies demonstrated that the parasite is affiliated with haplosporidians [20, 21] and belongs to the phylum of cercozoan [22].

Although methods for in vitro cultivation of the parasite are lacking, a protocol for parasite purification from infected oysters [23] enables in vitro studies on interactions between *B. ostreae* and its host cells, the haemocytes [24].

Three types of haemocytes were reported in the European flat oyster on the basis of morphological criteria: granulocytes (haemocytes with granules, diameter around 10 µm), large hyalinocytes (haemocytes without granules, diameter around 15 µm) and small hyalinocytes (haemocytes without granules, diameter from 5 to 10 µm) [19, 24, 25, 26, 27, 28].

Haemocytes play a pivotal role in mollusc defence against pathogens. The main cellular immune response consists of phagocytosis [29, 30]. Pathogens are internalised and then degraded through hydrolytic enzymes including esterases and production of reactive oxygen species (ROS) [28, 31, 32]. Esterases are enzymes belonging to the group of hydrolases catalysing the hydrolysis of ester bonds. The respiratory burst is a series of biochemical reactions that produce highly microbicidal ROS including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^+$). Although radicals produced during the respiratory burst are known to be involved in the destruction of parasites in different host species including molluscs [33, 34, 35], some intracellular parasites like *Leishmania sp* and *Perkinsus marinus* develop strategies to evade this process which allows them to invade and multiply within host cells [36, 37].

Some studies demonstrated that the parasite *Bonamia ostreae* was internalised after 30 minutes of contact with haemocytes and was not degraded after phagocytosis [24, 25]. Hervio et *al* [38], reported that the parasite possesses catalytic enzymes and acid phosphatase which could inhibit haemocyte activities developed for parasite degradation.

The current study based on flow cytometry was undertaken in order to further investigate mechanisms developed by *Bonamia ostreae* to survive within haemocytes. Haemocyte activities were monitored comparing cells in contact with live and dead parasites as well as haemocytes alone. Parasite internalisation inside haemocytes was also confirmed by light microscopy.

Cell mortality/viability, non specific esterase activities, respiratory burst and phagocytosis were monitored by flow cytometry in order to measure the impact of parasite on immune capacity of oyster haemocytes. Two different levels of parasite were used to test the effect of *Bonamia ostreae* on oyster haemocytes. Based on previous studies [24, 39] and on microscopic observation, two ratios were selected 5:1

and 10:1 parasites per haemocyte. In addition, both live and dead parasites were tested and compared in attempt to access the active or passive role played by the parasite in the modification of haemocyte activities.

## 2. Material and methods

### 2.1. Biological material

#### 2.1.1. Oysters

Two-year-old flat oysters *Ostrea edulis* were collected from Quiberon Bay (Brittany) in September 2007 and acclimatized in the quarantine facilities of Ifremer laboratory in La Tremblade (Charente-maritime, France) over 30 days. The oysters were maintained in 120 l raceways supplied with a constant flow of seawater enriched in phytoplankton (*Skeletonema costatum, Isochrisis galbana, Chaetoceros gracialis* and *Tetraselmis succica*).

#### 2.1.2. Haemolymph collection

Haemolymph was withdrawn from the adductor muscle sinus of 10 oysters using a 1 ml syringe equipped with a needle (0.40 mm x 90 mm). To eliminate debris the haemolymph samples were filtered through 60 µm nylon mesh and held on ice to prevent cell aggregation. The volume of haemolymph collected from each oyster was approximately 1 ml, samples were pooled, and haemocyte counts were performed using a Malassez-cell. The haemocyte concentration was adjusted to $5.10^5$ cells $ml^{-1}$ using 0.22 µm filtered sea water (FSW).

#### 2.1.3. Parasite purification

*Bonamia ostreae* was purified according to the protocol developed by Mialhe *et al.*(1988) [23]. Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After homogenisation of all the organs except the adductor muscle, parasites were concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient. Finally, the purified *B. ostreae* cells were suspended in FSW and counted using a Malassez-cell.

#### 2.1.4. Parasite inactivation

Parasites were inactivated at 100°C for 15 minutes and the suspensions of dead parasites were held on ice for 5 minutes.

### 2.2. In vitro infection protocol

Haemocytes ($5.10^5$ cells $ml^{-1}$) were incubated with live and dead parasites at two ratios (5:1 and 10:1 parasites per haemocyte) for two hours at 15°C. The control consisted of haemocytes alone suspended in FSW ($5.10^5$ cells $ml^{-1)}$. The experiments were performed in triplicate using three different pools of oysters.

### 2.3. Light microscopy

After two hours of incubation, 100 µl of each cell suspension were cytocentrifugated (100 × g, 4°C, 1 min), stained with Hemacolor® (Merck) and examined using light microscopy.

## 2.4. Flow cytometry analysis

For each sample, 5 000 events were counted using an EPICS XL 4 (Beckman Coulter). Based on size discrimination, parasites or other small particles were not counted, only haemocytes were taken into account for cell activity measures. Results were depicted as cell cytograms and reported in log scale fluorescence levels for each marker used. Fluorescence depended on the monitored parameters: non specific esterase activities, ROS production and phagocytosis were measured using green fluorescence while cell mortality was measured using red fluorescence.

### 2.4.1. Haemocyte mortality/viability

Haemocyte mortality was quantified using 200 µl of cell suspension. Cells were incubated in the dark for 30 minutes at 4°C with 10 µl of Propidium Iodide (PI, 1.0 $g.l^{-1}$, Interchim). PI is a fluorescent DNA/RNA-specific dye, which only permeates through the membranes of dead cells and stains the nucleic acids.

### 2.4.2. Non specific esterase activities

Non specific esterase activities were measured by adding 1 µl of a non specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes, 400 µmol $l^{-1}$) to 200 µl of a haemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and then transferred to ice for 5 minutes to stop the reaction.

### 2.4.3. ROS production

The respiratory burst was measured using the 2',7'-dichlorofluorescein diacetate (DCFH-DA), a non fluorescent fluorescein which permeates cells and is hydrolysed yielding hydrosoluble DCFH. In the presence of hydrogen peroxide, DCFH is oxided to DCF, a fluorescent product. Intracellular oxidation of DCFH is quantitatively related to oxidative burst in haemocytes. To measure ROS production, 2 µl of DCFH-DA (1mM) were added to 200 µl of the haemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and then transferred to ice for 5 minutes to stop the reaction.

### 2.4.4. Phagocytosis

Phagocytosis capacity of haemocytes was evaluated by using fluorescent beads [26]. Haemocyte suspension (200 µl) was incubated for one hour in the dark at room temperature with 10 µl of a 1/10 dilution of Fluorospheres carboxylate-modified microspheres (1µm diameter, Interchim).

## 2.5. Statistical analysis

An unilateral Test Z was performed to test superiority of the mean number of live vs dead parasites inside haemocytic cells.
Flow cytometry results were expressed as percentage of positive cells. In order to detect an effect of tested conditions, an ANOVA was performed using XLSTAT-Pro® version 7.5.3 software. Values were converted into r angular arcsinus $\sqrt{}$ (% of positive cells) before analysis to ensure the respect of *a priori* assumptions of normality and homogeneity. In the case of rejection of $H_0$, an *a posteriori* Tukey test was used to compare differences between means.

# 3. Results

## 3.1. Detection of parasites in haemocytes

Following two hours of in vitro contact between haemocytes and *Bonamia ostreae* (ratio 1:5), observations showed that both live and dead parasites were engulfed by the flat oyster haemocytes (Table 1). However, larger numbers of live parasites (average of 3.5 parasites per infected haemocyte) were detected in oyster haemocytes compared to dead parasites (average of 2 parasites per infected haemocyte)($p<0.05$). Parasites (live or dead) were only detected in large hyalinocytes and in granulocytes (Fig. 1) and not in small hyalinocytes.

## *3.2.* Haemocyte activities in the presence of *Bonamia ostreae*

Prior to haemocyte activity measurement, cell survival was checked by deducing cell mortality based on PI labelling. Whatever was the tested condition, percentage of live cells was never below 93.5%. Cell mortality was considered as equivalent between all tested conditions (Table 2).
For non specific esterase activities (Fig.2) and ROS (Fig.3), two different populations of cells were distinguished (population of negative cells producing no non specific esterase activities or no ROS and population of positive cells producing non specific esterase activities or ROS) according to the fluorescence intensity. For phagocytosis, populations were defined according to a protocol previously described [26, 40, 41] (Fig.4).

### 3.2.1. Non specific esterase activities

The percent of haemocytes positive for non specific esterase activities was 70.3 ± 13.7% in the control, 32.2 ± 1.8% after incubation with a 5:1 ratio of live parasites, 17.4 ± 3.4% after incubation with 10:1 ratio of live parasites, 41.9 ± 3.3% after incubation with a 5:1 ratio of dead parasites and 34.16 ± 10.3% after incubation with a 10:1 ratio of dead parasites, respectively (Fig.5). In presence of live and dead parasites (ratios 5:1 and 10:1), population of positive cells for esterase activities decreased significantly compared to the control ($p< 0.0001$). Live parasites at a ratio 10:1 induced a significantly more important decrease compared to live parasites at a ratio 5:1 and compared to dead parasites at ratios 5:1 and 10:1 ($p< 0. 001$).

### 3.2.2. ROS production

The percent of ROS positive haemocytes was 61 ± 19.8% in the control, 21.3 ± 4.1% after incubation with a 5:1 ratio of live parasites, 12.0 ± 2.5% after incubation with 10:1 ratio of live parasites, 33 ± 3.4% after incubation with a 5:1 ratio of dead parasites and 26.9 ± 4% after incubation with a 10:1 ratio of dead parasites, respectively (Fig.6). In presence of live and dead parasites (ratios 5:1 and 10:1), population of ROS positive cells decreased significantly compared to the control ($p< 0.0001$). Percentages of ROS positive cells for haemocytes in contact with live parasite at a 10:1 ratio were significantly different from those of haemocytes in presence of dead parasites at a ratio 5:1 ($p< 0.0001$) and at a ratio 10:1 ($p< 0.002$).

### 3.2.3. Phagocytosis assay

Comparison of the percent of haemocytes positive for phagocytosis showed only minor differences between controls and the tested conditions (Fig.7). Regardless of test

conditions, 30% to 40% of the haemocytes engulfed 3 or more than 3 beads after 2 hours.

## 4. Discussion

Although previous studies based on flow cytometry aimed at describing haemocyte morphology and activities in oysters [26, 42, 43], abalone [44] or mussels [45], flow cytometry has rarely been used to investigate interactions between molluscs and pathogens, more specifically pathogens infecting haemocytes [40, 46, 47, 48].

In the present study, flow cytometry and light microscopy were used to determine the effects of both live and dead *Bonamia ostreae* at two different ratios on haemocyte parameters of flat oysters *Ostrea edulis*. The tested ratios 5 parasites and 10 parasites per haemocyte were chosen based on previous studies [24, 39] and are expected to reflect the conditions of haemocytes exposed under high level *in vivo* infections. In vitro assays were conducted by incubating haemocytes with purified parasites for 2 hours at 15°C. The temperature and the incubation period were selected according to previous studies [24, 25, 49, 50]. In a previous study after a contact of 30 minutes, parasites were engulfed by haemocytes, and after four hours, vacuoles containing *B. ostreae* were observed within haemocytes [51]. A 2 hour incubation was deemed sufficient to measure effects of the parasite on post phagocytosis mechanisms. Microscopic examination of cytocentrifuged cells confirmed that both live and dead parasites were engulfed by haemocyte but not destroyed after two hour of incubation [24]. Internalisation of dead parasites suggests that the process is mediated by haemocytes themselves. Chagot et *al* [25] demonstrated that parasites were not engulfed when haemocytes were treated with cytochalasin B prior parasite contact. These results support an active participation of host cells in the internalisation of the parasite. However, the same study, [25] demonstrated that the treatment of parasites with cytochalasin B prior to cell contact induced a significant decrease of parasite internalisation. These results also suggest that the parasite may play an active role for its own internalisation in oyster haemocytes. In our study, higher numbers of live parasites were detected in oyster haemocytes compared to dead parasites confirming that the entry of the parasite in haemocyte may also mediated by the parasite itself.

Cell mortality did not show an increase in haemocyte mortality after contact with dead or live parasites. This result suggests that *Bonamia ostreae* does not kill haemocytes soon after internalisation and that the conditions used in our experiments did not affect the viability of haemocytes.

In order to investigate interactions between *Bonamia ostreae* and haemocytes, we measured non specific esterase activities and ROS. Although non specific esterase activities were recognized as reflecting the global activity of haemocytes, esterases may be also involved in degradation of internalised particles after phagocytosis. We also tested the ability of haemocytes to phagocyte live and dead parasites. These parameters were previously used to measure the effects of abiotic factors including temperature or salinity [40, 52] or biotic factors [48] on haemocyte activities.

The presence of parasites significantly modified non specific esterase activities of haemocytes. Live parasites induced a decrease in percentage of non specific esterase activities positive cells. Although dead parasites modified the percentage of non specific esterase activities positive haemocytes when present at a 10:1 ratio, it was at a lesser extent when compared to live parasites. These results, in addition to the lack of haemocyte mortality, suggest a direct impact of parasites on host cell esterase activities. Catalytic enzymes present in *Bonamia ostreae* [38] might be able to inhibit hydrolases (ie esterases) produced by haemocytes.

Similarly, the presence of *Bonamia ostreae*, (particularly live parasites) significantly modified ROS detection in haemocytes. In all test conditions the percentage of ROS

positive haemocytes decreased after two hours of incubation with parasites. The decrease was significantly more pronounced in presence of live *B. ostreae* compared to dead ones. As no cell mortality was detected, parasites may have a direct impact on haemocyte ROS production. The inhibition of oxygen radical production facilitates intracellular survival of protozoan parasites including *Trypanosoma sp* [53], *Toxoplasma sp* [54, 55] and *Leishmania sp* [56].
A study carried out to describe interactions between haemocytes from *Crassostrea virginica* and the parasite *Perkinsus marinus* showed that live parasites induced an inhibition of ROS release from oyster haemocytes whereas heat killed parasites did not modify ROS production [57]. Inhibition of ROS production was also reported in haemocytes from *Pecten maximus* incubated with live Rickettsiales-like organisms [58]. *Perkinsus marinus* and Rickettsiales-like organisms possess acid phosphatase, partly responsible for the suppression or inhibition of ROS production [58, 59]. Hervio et al [38] demonstrated the presence of acid phosphatase activity in haplosporosomes of *Bonamia ostreae*. Acid phosphatase can inactivate the production of NAD(P)H oxidase and thus prevents ROS production. The level of acid phosphatase activity in *B. ostreae* is equivalent [38] to levels found in several Leishmania species [60] in which the inhibitor role of the NAD(P)H oxidase has been shown.
Latex beads were used to measure the phagocytosis ability of haemocytes following 2 hours of incubation with parasites. Phagocytosis measurements showed little variation between the control (haemocytes alone) and the tested conditions, suggesting that neither live or dead parasites stimulate or inhibit phagocytosis ability of haemocytes.
Phagocytosis plays an important role in interactions between host and pathogens. Many intracellular parasites stimulate their own phagocytosis through activation of receptors or production of actin filaments. In our study, phagocytosis ability of haemocytes against inert material like latex beads was not affected by the presence of parasites. However, the use of bacteria could better reflect response of haemocytes against pathogenic organisms. Besides, recent tests were performed to measure latex bead phagocytosis in presence of parasites by using a higher size discriminant than the one used in the present study. This protocol modification allowed to observe a decrease of phagocytic ability of haemocytes after 4 hours of incubation with parasites (Morga, pers com).

In conclusion, the present study investigated the effect of the parasite *Bonamia ostreae* on haemocyte activities using flow cytometry after two hours of in vitro incubation. Light microscopy confirmed that live and dead parasites were engulfed by the haemocytes after two hours of contact. After its entry into the haemocyte, the parasite induced changes within the host cell including a modification of cellular activities necessary to escape the degradation by haemocytes. These results contribute to a better understanding of the interactions between *B. ostreae* and haemocytes and more specifically the effect of the parasite on the host cell to ensure its survival and its ability to multiply within the cell.

## Acknowledgement

The authors are very grateful to Dr. Gary Meyer (Pacific Biological Station, Nanaimo, Canada) for revising the manuscript. The Region of Poitou Charente and IFREMER supported this research. The authors thank Raphael Brizard and his team for technical assistance for the maintenance of oysters in raceways in Ifremer La Tremblade quarantine.

## References


[1] Comps M. Observation sur les causes d'une mortalité anormale des huîtres plates *Ostrea edulis* dans le bassin de Marennes. Rev Trav Inst Pêches Marit. 1970;34(3):317-26.

[2] Herrbach B. Sur une affection parasitaire de la glande digestive de l'huître plate *Ostrea edulis*. Rev Trav Inst Pêches Marit. 1971;35(1):79-87.

[3] Grizel H, Comps M, Bonami JR, Cousserans F, Duthoit JL, Le Pennec MA. Recherche de l'agent de la maladie de la glande digestive de *Ostrea edulis*. Science et pêche. Bull Inst Pêches Marit. 1974;240:7-30.

[4] Pichot Y, Comps M, Tige G, Grizel H, Rabouin MA. Research on *Bonamia ostreae* gen. n., sp. n., a new parasite of the flat oyster *Ostrea edulis* L. Rev Trav Inst Pêches Marit. 1979;43(1):131-40.

[5] Comps M, Tige G, Grizel H. Etude ultrastructurale d'un protiste parasite de l'huître *Ostrea edulis* L. C. R. Acad Science Paris, 1980.290, Série D:383-5.

[6] Comps M. Recherches histologiques et cytologiques sur les infections intracellulaires des mollusques bivalves marins. Thèse de doctorat d'état en sciences naturelles, Université de Montpellier. 1983.

[7] Van Banning P. Control of *Bonamia* in Dutch oyster culture. In: Ellis AE, editor. Fish and shellfish pathology, Proceedings of a Symposium, 20-23 September 1983, Plymouth. London: Academic Press; 1985. p. 393-6.

[8] Van Banning P. Observations on bonamiasis in the stock of the European flat oyster, *Ostrea edulis*, in the Netherlands, with special reference to the recent developments in Lake Grevelingen. Aquaculture. 1991;93(3):205-11.

[9] Elston RA, Kent ML, Wilkinson MT. Resistance of *Ostrea edulis* to *Bonamia ostreae* infection. Aquaculture. 1987;64(3):237-42.

[10] Montes J. Development of *Bonamia ostreae* parasitosis of flat oyster, *Ostrea edulis*, from Galicia, northwest Spain. In: Perkins FO, Cheng TC, editors. Pathology in Marine Science. San Diego CA (USA): Aacademic Press; 1990. p. 223-7.

[11] Bucke D, Hepper B. *Bonamia ostreae* infecting *Ostrea lutaria* in the UK. Bull Eur Ass Fish Pathol. 1987;7(3):79-80.

[12] Rogan E, Culloty SC, Corss TF, Mulcahy MF. The detection of *Bonamia ostreae* (Pichot et *al*. 1980) in frozen oysters (*Ostrea edulis* L.) and the effect of the parasite on condition. Aquaculture. 1991;97(4): 311-5.

[13] Lynch SA, Armitage DV, Wylde S, Mulcahy MF, Culloty SC. The susceptibility of young prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae*. J. Shellfish Res. 2005;24(4):1019-25.

[14] Balouet G, Poder M, Cahour A. Haemocytic parasitosis : morphology and pathology of lesions in the French flat oyster, *Ostrea edulis* L. Aquaculture. 1983;34:1-14.

[15] Caceres-Martinez J, Robledo JAF, Figueras A. Presence of *Bonamia* and its relation to age, growth rates and gonadal development of the flat oyster, *Ostrea edulis*, in the Ria de Vigo, Galicia (NW Spain). Aquaculture. 1995;130(2):15-23.

[16] Culloty SC, Mulcahy MF. Season-, age-, and sex-related variation in the prevalence of bonamiasis in flat oysters (*Ostrea edulis* L.) on the South coast of Ireland. Aquaculture. 1996;144(1-3):53-63.

[17] Grizel H. Etude des récentes épizooties de l'huître plate *Ostrea edulis* Linné et de leur impact sur l'ostréiculture bretonne. Thèse de doctorat d'Etat en Sciences Naturelles, Université de Monpellier. 1985.

[18] Montes J, Anadon R, Azevedo C. A possible life cycle for *Bonamia ostreae* on the basis of electron microscopy studies. J Invertebr Pathol. 1994;63(1):1-6.

[19] Cochennec-Laureau N, Auffret M, Renault T, Langlade A. Changes in circulating and tissue-infiltrating hemocyte parameters of European flat oysters, *Ostrea edulis*, naturally infected with *Bonamia ostreae*. J Invertebr Pathol. 2003 83(1):23-30.

[20] Cochennec N, Le Roux F, Berthe F, Gerard A. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J Invertebr Pathol. 2000 76(1):26-32.
[21] Carnegie RB, Barber BJ, Culloty SC, Figueras AJ, Distel DL. Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia. Dis Aquat Org. 2000 28;42(3):199-206.
[22] Cavalier-Smith T, Chao EE. Phylogeny and classification of phylum Cercozoa (Protozoa). Protist. 2003 154(3-4):341-58.
[23] Mialhe E, Bachere E, Chagot D, Grizel H. Isolation and purification of the protozoan *Bonamia ostreae* (Pichot et al. 1980), a parasite affecting the flat oyster *Ostrea edulis* L. Aquaculture. 1988;71(4):293-9.
[24] Mourton C, Boulo V, Chagot D, Hervio D, Bachere E, Mialhe E, et al. Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca: Bivalvia): *In vitro* system establishment. J Invertebr Pathol 1992.;59(3): 235-40.
[25] Chagot D, Boulo V, Hervio D, Mialhe E, Bachere E, Mourton C, et al. Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas (*Mollusca: Bivalvia): Entry mechanisms. J Invertebr Pathol. 1992;59(3): 241-9.
[26] Xue Q-G, Renault T, Chilmonczyk S. Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. Fish Shellfish Immunol. 2001;11(7):557-67.
[27] Auffret M. Morphologie comparative des types hémocytaires chez quelques mollusques bivalves d'intérêt commercial. Thèse de 3ème cycle Université de Bretagne Occidentale. 1985
[28] Bachère E, Hervio, D. , Mialhe, E. Luminol dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. Dis Aquat Org. 1991;11;173-80.
[29] Cheng TC. Invertebrate blood Cells. Academic press of London. 1981:p 233-99.
[30] Fisher SW. Structure and functions of oyster hemocytes. In Brehélin M., editor. Immunity in invertebrates. 1986;3:25-35.
[31] Pipe RK, Porte C, Livingstone DR. Antioxidant enzymes associated with the blood cells and haemolymph of the mussel *Mytilus edulis.* Fish Shellfish Immunol. 1993;3(3):221-33.
[32] Carballal MJ, López C, Azevedo C, Villalba A. Enzymes Involved in Defense Functions of Hemocytes of Mussel *Mytilus galloprovincialis.* J Invertebr Pathol. 1997;70(2):96-105.
[33] Toreilles J, Guerin MC, Roch P. Reactive oxygen species and defense mechanisms in marine bivalves. C. R. Acad Science Paris, série III.; science de la vie ;1996 (319):209-18.
[34] Humphries JE, Yoshino TP. Regulation of hydrogen peroxide release in circulating hemocytes of the planorbid snail *Biomphalaria glabrata.* Dev Comp Immunol. 2008;32(5):554-62.
[35] Hahn UK BR, Bayne CJ. Killing of *Schistosoma mansoni* sporocysts by hemocytes from resistant *Biomphalaria glabrata* : Role of reactive oxygen species. J Parasitol 2001;87: 292-9
[36] Dermine J-F, Desjardins M. Survival of intracellular pathogens within macrophages. Protoplasma 1999;210:11-24
[37] Schott EJ, Pecher WT, Okafor Florence, and Vasta GR The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. Exp Parasitol 2003;105: 232-40
[38] Hervio D, Chagot D, Godin P, Grizel H, Mialhe E. Localization and characterization of acid phosphatase activity in *Bonamia ostreae* (Ascetospora), an intrahemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia). Dis Aquat Org. 1991;12(1): 67-70.

[39] Cochennec N. *Bonamia ostreae*, parasite de l'huître plate, *Ostrea edulis*, sa position taxonomique parmi les parasites du groupe "Microcell," analyses des interactions hôte/parasite chez plusieurs populations d'huîtres plates. Thèse de Doctorat. Biologie cellulaire, Université de la Rochelle, France. 2001.
[40] Gagnaire B, Frouin H, Moreau K, Thomas-Guyon H, Renault T. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish Shellfish Immunol. 2006;20(4):536-47.
[41] Hégaret H, Wikfors GH, Soudant P. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation: II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. J Exp Mar Biol Ecol. 2003;293(2):249-65.
[42] Allam B, Ashton-Alcox KA, Ford SE. Flow cytometric comparison of haemocytes from three species of bivalve molluscs. Fish Shellfish Immunol. 2002;13(2):141-58.
[43] Goedken M, De Guise S. Flow cytometry as a tool to quantify oyster defence mechanisms. Fish Shellfish Immunology. 2004;16(4):539-52.
[44] Travers M-A, Mirella da Silva P, Le Goïc N, Marie D, Donval A, Huchette S, et al. Morphologic, cytometric and functional characterisation of abalone (*Haliotis tuberculata*) haemocytes. Fish Shellfish Immunol. 2008;24(4):400-11.
[45] Soares-da-Silva IM, Ribeiro J, Valongo C, Pinto R, Vilanova M, Bleher R, et al. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. Comp Biochem and Physiol A.. 2002. 132:541-53.
[46] Lambert C, Soudant P, Choquet G, Paillard C. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish Shellfish Immunol. 2003;15(3):225-40.
[47] García-García E, Prado-Álvarez M, Novoa B, Figueras A, Rosales C. Immune responses of mussel hemocyte subpopulations are differentially regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. Dev Comp Immunol. 2008;32(6):637-53.
[48] Da Silva PM, Hégaret H, Lambert C, Wikfors GH, Le Goïc N, Shumway SE, et al. Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions. Toxicon. 2008;51(4):563-73.
[49] Fisher WS, Auffret M, Balouet G. Response of European flat oyster (*Ostrea edulis*) hemocytes to acute salinity and temperature changes. Aquaculture. 1987;67(1-2):179-90.
[50] Fisher WS. *In vitro* binding of parasites (*Bonamia ostreae*) and latex particles by hemocytes of susceptible and insusceptible oysters. Dev Comp Immunol. 1988 ;12(1):43-53.
[51] Chagot, D."Caractérisation morphologique et fonctionnelle des hémocytes d'Ostrea edulis et Crassostrea gigas, mollusques bivalves. Etude in vitro de leurs interactions avec le protozoaire Bonamia ostreae (Ascetospora). Thèse du diplôme de l'école Pratique des Hautes Etudes de Montpellier. 1989.
[52] Sauvé S, Hendawi M, Brousseau P, Fournier M. Phagocytic Response of Terrestrial and Aquatic Invertebrates Following *in Vitro* Exposure to Trace Elements. Ecotoxicol Environ Saf. 2002;52(1):21-9.
[53] Penketh PG, Kennedy WPK, Patton CL Sartorelli. AC,. Trypanosomatid hydrogen peroxyde metabolism. FEBS letter. 1987;221(2):427-31.
[54] Murray HW, Nathan, CF, Cohn ZA. Macrophage oxygen-dependent antimicrobial activity. J Exp med. 1980;152:1610-24.
[55] Shrestha SP, Tomita T, Weiss LM, Orlofsky A. Proliferation of *Toxoplasma gondii* in inflammatory macrophages in vivo is associated with diminished oxygen radical production in the host cell. Int J Parasitol. 2006 ;36(4):433-41.
[56] Murray HW. Susceptibily of leishmania to oxygen intermediate and killing by normal macrophage. J Exp med. 1981;153(1302-1315).

[57] Volety AK, Chu FLE Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*. Dev Comp Immunol. 199519: 135-42
[58] Le Gall G, Miahle E, Chagot D, Grizel H. Epizootiological study of rickettsiosis of the Saint-Jacques scallop *Pecten maximus*. Dis Aquat Org. 1991;10:139-45.
[59] Volety AK, Chu F-LE. Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the american oyster, *Crassostrea virginica*. J Parasitol 1997;83(6):1093-8.
[60] Lovelace JK, Gottlieb M. Effect of tunicamycin on the extracellular acid phosphatase of *Leishmania donovani* promastigotes. Mol Biochem Parasitol. 1987;22(1):19-28.

## Tables

| | Haemocytes with live parasites | Haemocytes with dead parasites |
|---|---|---|
| **5. Total haemocyte counts** | 120 | 110 |
| 5.1. Granulocytes % (n) | 35% (42) | 29% (32) |
| 5.2. Infected granulocytes | 72% (31) | 71% (25) |
| 5.3. Large hyalinocytes % (n) | 45% (55) | 50% (55) |
| 5.4. Infected large hyalinocytes | 27% (12) | 28% (10) |
| 5.5. Small hyalinocytes % (n) | 19% (23) | 20% (23) |
| Infected small hyalinocytes | 0 | 0 |
| Parasites average by infected haemocytes ±Standard error | 3.5 ± 0.6 | 2 ± 0.34 |

**Table 1**

Differential haemocyte counts and percentage of infected cells by haemocyte type (granulocyte, large and small hyalinocytes) following two hours of incubation with live and dead parasites (ratio 1:5).

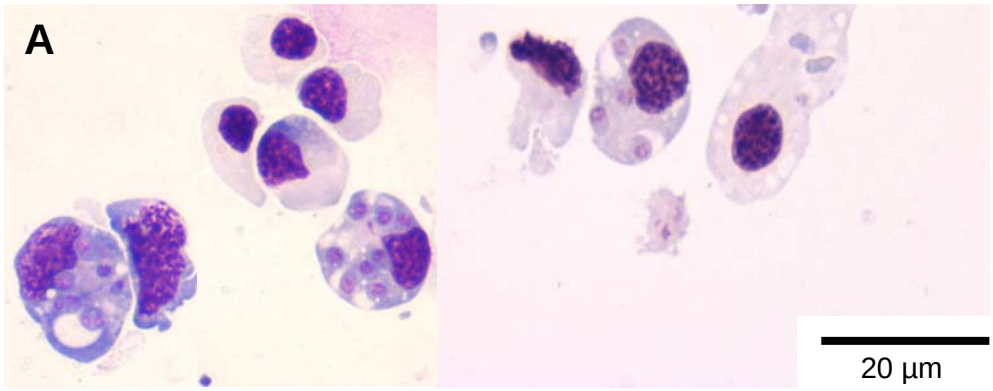
| | Control | Haemocytes+ parasites (5 :1) | Haemocytes+ parasites (10:1) | Haemocytes+ dead parasites (5:1) | Haemocytes+ dead parasites (10:1) |
|---|---|---|---|---|---|
| % of viable cells | 96% | 97.1% | 97% | 95% | 93.5% |
| SD | ±0.5 | ±0.5 | ±0.6 | ±0.4 | ±2 |

**Table 2**

Percentages of viable haemocytes after two hours of incubation with live and dead parasites at 5:1 and 10:1 ratios. Control = haemocytes alone. Values are means of three replicates and corresponds to standard deviation.

## Figures

**Fig 1**



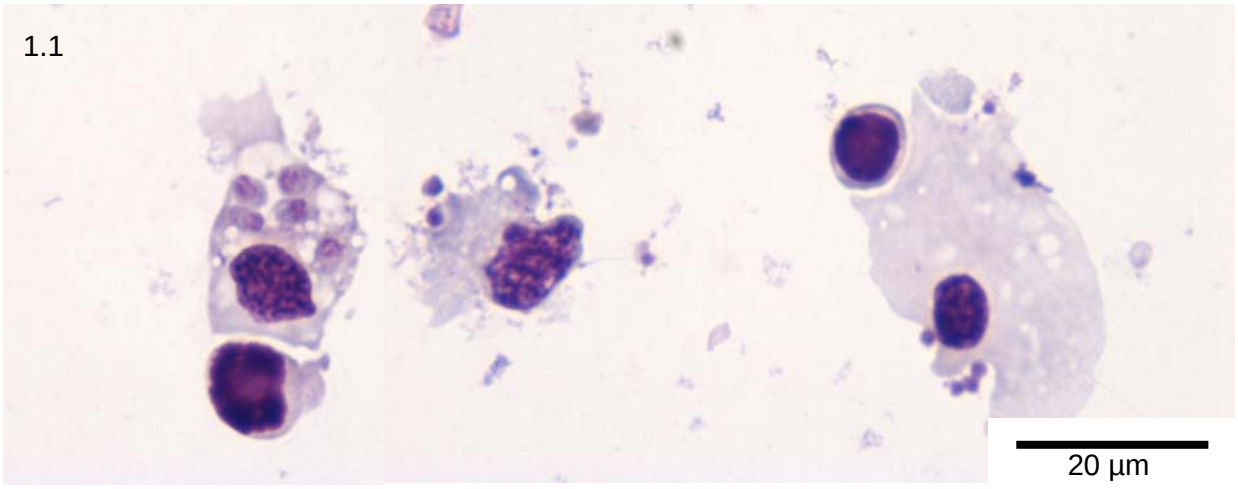




Fig 1 : Haemocytes from *Ostrea edulis* stained with Hemacolor® Merck following cytocentrifugation (A) Haemocytes after 2 hours incubation with live *Bonamia ostreae* (B) Haemocytes after 2 hours incubation with dead *Bonamia ostreae*.

**Fig 2**

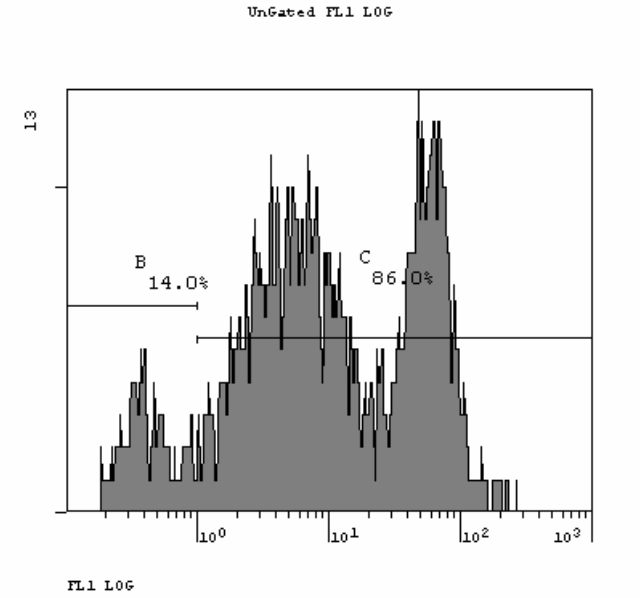



Fig 2: Cytogram of cells stained with FDA, substrate used to measure non specific esterase activities. Horizontal-axis, intensity of fluorescence; Vertical axis, number of cells: (B) population of cells negative for non specific esterase activities; (C) population of cells positive for non specific esterase activities.

**Fig 3**

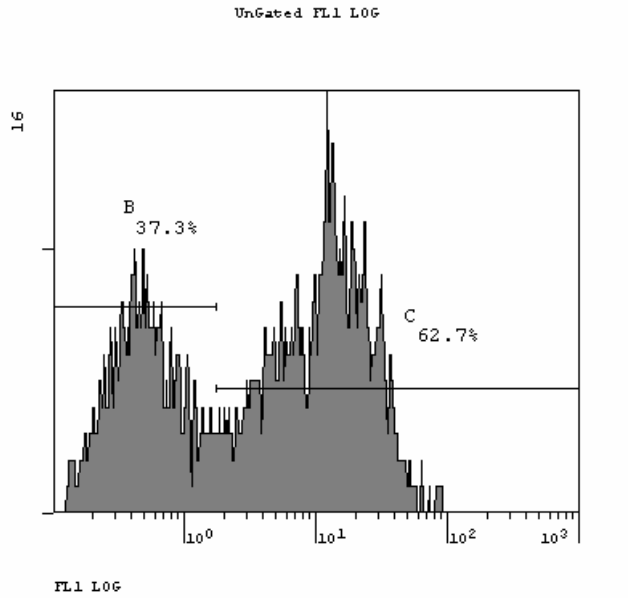



Fig 3: Cytogram of cells stained with DCFH-DA, substrate used to measure reactive oxygen species (ROS). Horizontal-axis, intensity of fluorescence; Vertical axis, number of cells: (B) population of cells negative for ROS; (C) population of cells positive for ROS.

**Fig 4**

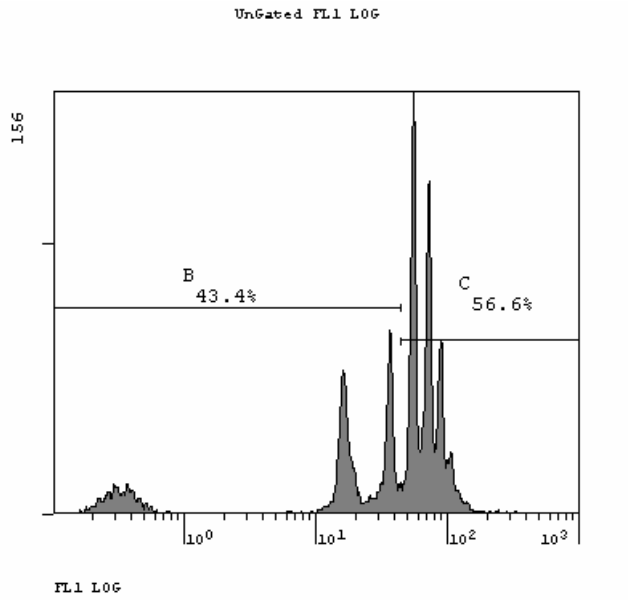



Fig 4: Cytogram of cells incubated with fluorescent beads. Horizontal-axis, intensity of fluorescence; Vertical axis, number of cells: (B) population of cells which engulfed 0 to 2 beads; (C) population of cells which engulfed 2 or more beads.

**Fig 5**

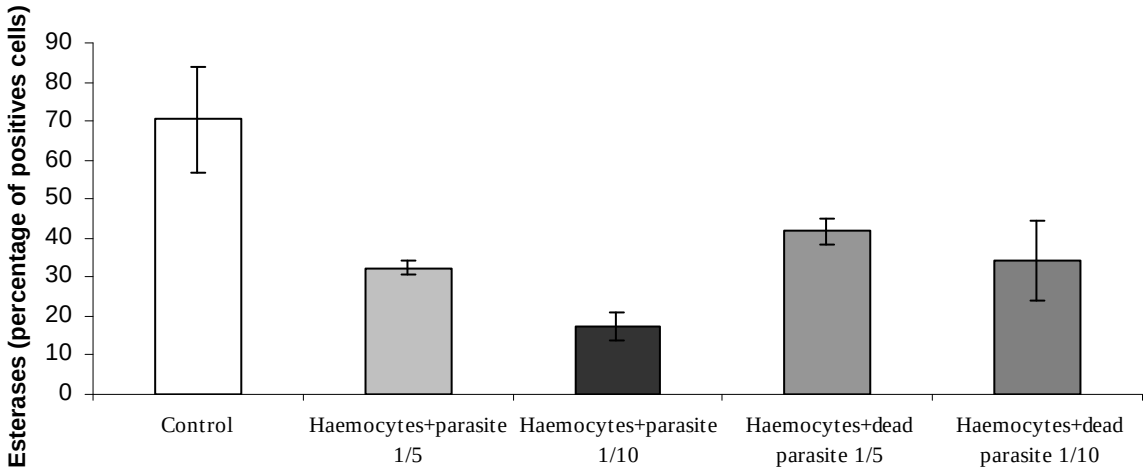



Fig 5: Percentage of haemocytes positive for non specific esterase activity after two hours of incubation with live and dead parasites at 5:1 and 10:1 ratios. Control = haemocytes alone. Values are means of three replicates and bars represent standard deviation.

**Fig 6**

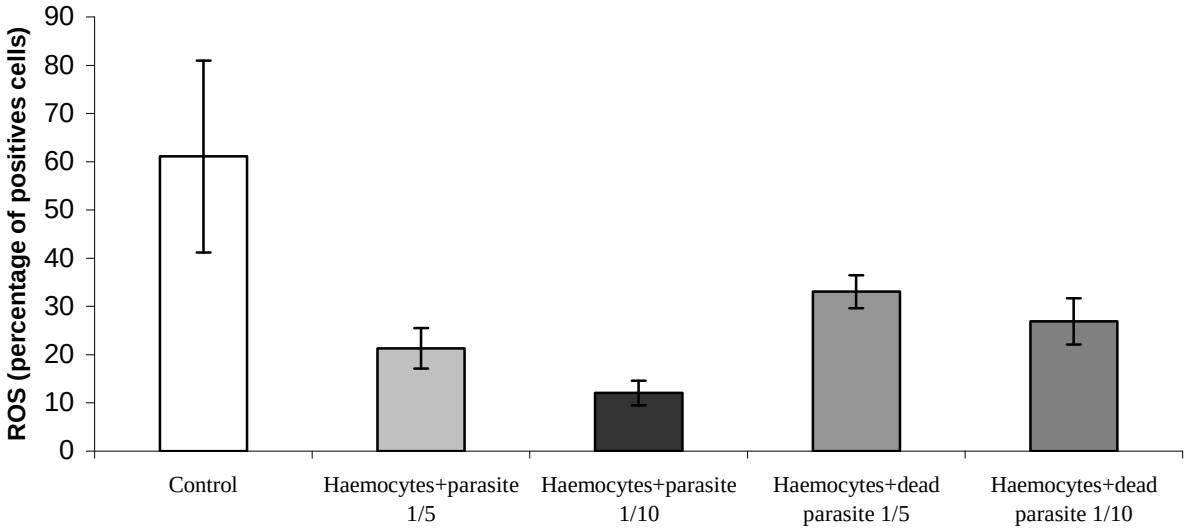



Fig 6 : Percentage of haemocytes positive for ROS after two hours of incubation with live and dead parasites at 5:1 and 10:1 ratios. Values are means of three replicates and bars represent standard deviation.

**Fig 7**

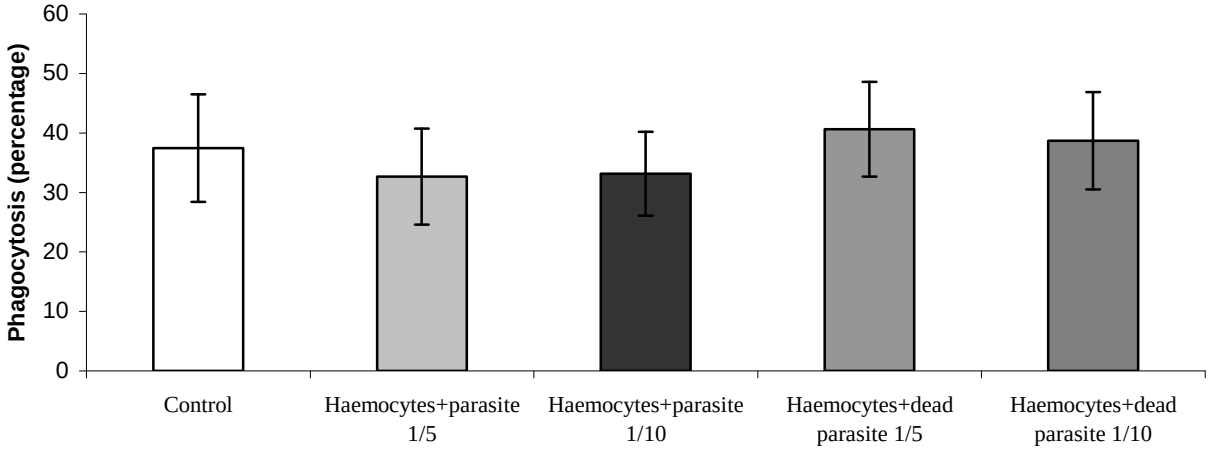



Fig 7 : Percentage of haemocytes positive for phagocytosis after two hours of incubation with live and dead parasites at 5:1 and 10:1 ratios. Values are means of three replicates and bars represent standard deviation.