SUP biotech PARIS

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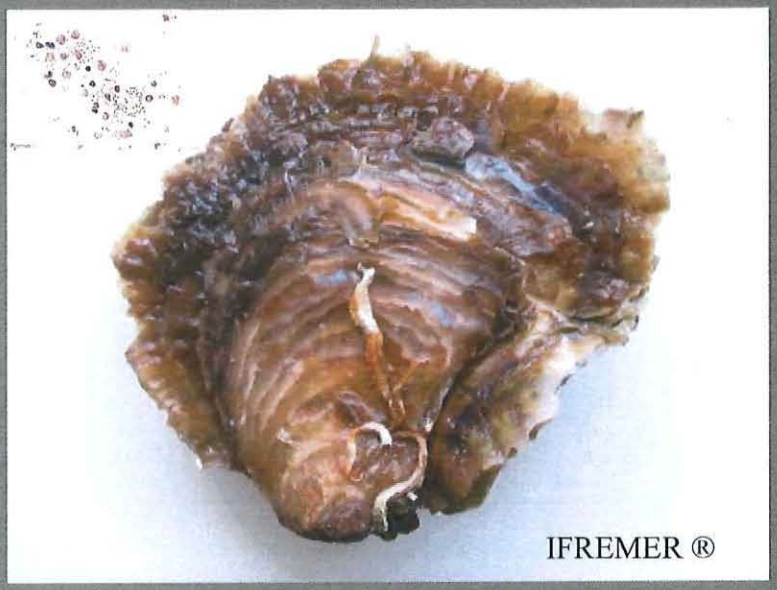
Report of Internship 2008

Quantification of genes expressed during an *in vitro* infection of haemocytes from *Ostrea edulis* with parasites *Bonamia ostreae* by Real times PCR.



IFREMER ®

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Presented by Amélie SEGARRA
IFREMER

25 février au 23 mai 2008

Station

Dépendant du centre de Nantes, située au cœur du bassin ostréicole de Marennes-Oléron, la station **IFREMER** de La Tremblade abrite deux laboratoires, celui de l'Environnement Ressources des Pertuis Charentais qui assure la gestion des différents réseaux de surveillance et le Laboratoire de Génétique et Pathologie (LGP).

Le stage a été effectué au sein du **Laboratoire Génétique et Pathologie (LGP)** à La Tremblade (17).

La pathologie :

Surveillance des ressources conchylicoles, l'identification des agents pathogènes, la description de leur cycle de développement, la mise au point des techniques de reproduction expérimentale des maladies, le développement d'outils performants de diagnostic utilisables à des fins de recherche ou de contrôle, l'étude de l'impact de ces maladies et de leur évolution géographique et temporelle.

La génétique :

Etude des ressources génétiques, test de nouvelles espèces de mollusques, de nouvelles populations et d'hybrides pour limiter les risques liés à la monoculture. Obtention de souches résistantes ou tolérantes aux maladies pour essayer d'apporter des réponses aux épizooties qui remettent en cause les productions.

Création de souches ou de lignées présentant de meilleures performances de croissance, de qualité de chair, une meilleure adaptation aux conditions de milieu d'élevage ou éventuellement de faibles besoins métaboliques, pour améliorer la productivité des entreprises.

En tant que laboratoire thématique, le **LGP** anime les programmes de recherche en génétique et pathologie ainsi que le réseau de surveillance en pathologie des mollusques (**REPAMO**).

En pathologie, le **LGP** est également **Laboratoire Communautaire de Référence** (**LCR**) pour l'Union Européenne pour l'étude des maladies des mollusques et **laboratoire de référence** pour l'**OIE** (*Office International des Epizooties*) pour la bonamiose et la marteiliose.

Abreviation list

ARP: Actin regulator polymerisation

cDNA :complementary Deoxyribonucleic acid

Ct : Threshold cycle

DNA: Deoxyribonucleic acid

F: foward

FSSW: filter-sterilized seawater

LGP: laboratory genetic and pathology

MAPK: Mito antigen protein kinease

NTC : no template control

OGST: Oméga gluthatione s transférase.

OIE : world organization for animal health

P450: cytochrome P450

R: reverse

Rcf: Relative centrifugal force

RNA : Ribonucleic acid

ROS: Reactive oxygen species

RT-PCR: reverse transcriptase Polymerase chain reaction

SSH: Subtractive Suppressive Hybridization

TIMP: Tissu Inhibitor of MetalloProtease

Q PCR: real time quantitative Polymerase chain reaction

SOMMAIRE

Introduction

Oyster farming represents a significant economic part of the French aquaculture production. This production is approximately about 150 000 tons of shells a year [Buestel, 2004]. However the development of this activity can be limited by infectious diseases (virus, protozoan or bacteria).

Flat oysters suffered from two epizooties caused by two parasitic diseases: marteilliosis and the bonamiosis. These diseases appeared at the end of the Sixties and Seventies respectively. These diseases had a negative impact on the economy because they decrease the production of Flat oysters by 90% between 1979 and .1980. [Buestel 2004, Grizel 1985]

The means of fights against bonamiosis are limited and mainly rely on a preventive approach. In fact, it is impossible to use the vaccination especially because they do not have abodes. But it is also impossible to use treatments for invertebrate because they are product in open systems on the wild. The bonamiosis is a notifiable disease listed by the The World Organisation for Animal Health (OIE) as well as by European Union. That implies a surveillance of stocks and restrictions of transfer in order to avoid the contamination of healthy zones free. In addition another approach is based on the obtention of resistant animals through a genetic selection. Thanks to that it is possible to obtain a better rate of survival in the zones where the parasite is endemic [Naciri *et Al.*; 1998].

In this context, a better understanding of defence mechanisms developed by the flat oyster against parasite and interaction between oyster and parasite is needed. These data are necessary for the conservation of the flat oyster and to the restoring of flat oyster on our coasts.

The objective of this work was to study *in vitro* the expression of genes of haemocytes of flat oyster in contact with some purified parasite *Bonamia ostreae*. In a first step, our study has consisted in standardizing the Real Time Quantitative PCR in order to obtain curves standards for quantity the expression of genes of *Ostrea edulis*.

After a session dedicated to bibliographical researches, we will see material and methods presented in the second part. Lastly results will be exposed and discussed.

I. State of the art

From Roman antiquity until nowadays, oysters have always been considered as a well appreciated product. In the 18th century, the oyster consumption was more and more successful. Consequently, the resources became exhausted. To mitigate this deficit, the culture of oysters gradually becomes necessary to the next century. In the 19th century, the creation of the first parks was born in the Basin of Arcachon under Napoleon III.

At that time, the flat oyster (*Ostrea edulis*) was the only species present on the French littoral. French State dealt with the management of the coasts and laid down rules aiming at managing this activity. In spite of these measurements and the possibility of collecting the spat oyster, the production of flat oysters remains insufficient compared to the demand. In 1857 the government authorized the import of oyster from Portugal. In 1868, the Portuguese oyster *Crassostrea angulata* started to its proliferete in particular because of the incident of the boat "Morlaisien". This last was obliged to discard a part of the oysters in the estuary of the Gironde during storm.

Epizooty decimated the flat oyster, which became rare and was replaced by cupped Portuguese oysters. However first epizooty caused by an iridovirus called "disease of the gills" decimated Portuguese oyster in the 70ies which were replaced by *Crassostrea gigas.* Indeed *Crassostrea gigas* was imported from Japan and USA in order to start again the production of oyster in French. Before 1970 bonamiosis and marteiliosis, another protozoan disease appeared, drastically reduced the French flat oyster production from 20 000 tonnes in 1970 to less than 2000 tonnes after 1981. (*Figure 1*) [Grizel 1985, Heral and Goulletquer 1997]

The economic production essentially depend on *Crassostrea gigas.*

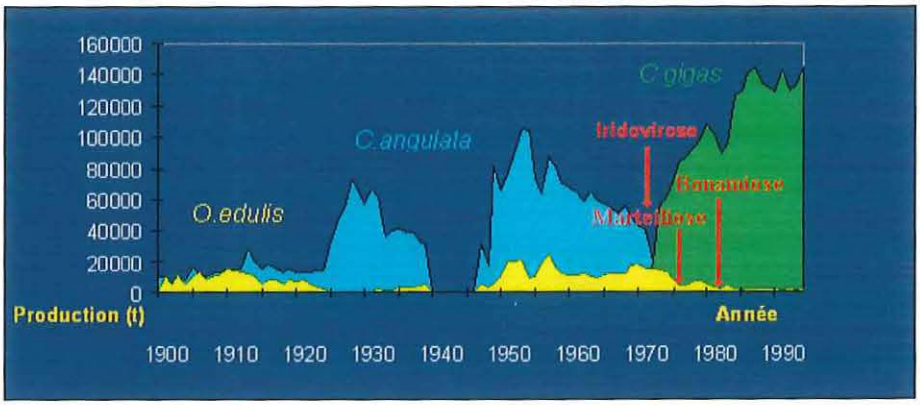


Figure 1: French Production of oysters (1900 to 2000) (*Ostrea edulis, C.angulata, C.gigas*).

The culture of oyster is essential for the economy of the concerned areas. This industry field allows the creation of 2500 seasonal employment and more then 6000 employment all the year. It is principally at Christmas than 75% of the annual sales are carried out.

The world production of oysters is estimated at 3.25 million tons in 2005 by FAO, Food and Agriculture Organization. France is the first producer in Europe with 137.000 tons in 1998-99 and the fourth global after the USA, Japan and Korea. *Appendix1* .

Currently in France, oyster farms are found on the Atlantic littoral, near in the English Channel and also in the Mediterranean Sea.

I. 1 Flat oyster: *Ostrea edulis*

In France, the production of flat oyster *Ostrea edulis* is essentially takes place in Brittany. 99% of spat is collected from the wild. The bay of Quiberon (Morbihan) ensures 87.8 % of the production of spat in France against 12.2 % at Brest (Finistere). *Appendix 2.* When oysters are 1 month, part of them is transferred to Cancale (Coasts of Armor), for growing before being marketed. 50% flat oysters are selling from Belon between 2 and 3 years old. Their size is 8 cm. [Girard *et al.*; 2005].

a) Systematic and phylogeny

The flat oyster is a bivalve mollusc of belonging to family of *Ostreidae.* (Table 1 and *Appendix 3.*)

Kingdom:	Animal
Sub-kingdom:	Mollusc
Class :	Bivalve
Order :	*Filibranchia*
Suborder :	*Anisomyaria*
Family :	*Ostreidae*
Genus :	*Ostrea*
Species :	*Ostrea edulis*

Table 1: Systematic of *Ostrea edulis* [Blanchet 2005]

b) Way of life and biology

In general a low salinity causes the death of oyster. However, many species can remain during 6 months in reduced salinities and of strong variations in temperature generate loss. The oyster requires oxygenated water and rich in plankton. Flat oyster is viviparous, fecundation is done inside the shell and larvae are rejected outside the shell after 4days. The flat oyster is hermaphrodite and can produce 1 million eggs and several tens of million spermatozoa each year. In May, the spherical egg is 0.1 mm in diameter. Spermatozoa are collected by gills of "female" oyster. They penetrate in the channels of genital gland where they fertilize eggs. After the eggs are expelled between gills layers of oyster where they will remain eight days before the larvae are not released definitively. Adult nourishes of plankton (phytoplankton and of zooplankton). Gills of flat oyster allow the filtration of water. On average 2 liters of sea water are filtered per hour [Anonymous 1].

c) Anatomy

The shell of *Ostrea edulis* is round presenting two unequal valves. The left valve is more concave and contains the flesh of oyster. The mantle is similar to a fine veil of flesh which ensures the growth and the development of the shell of mollusk. It also contributes to the production of the mother-of-pearl which covers the inner shell. The hinge orders the opening of oyster while the adductor muscle maintains shell closed. The gills have two distinct roles: breathing and feeding by filtering and conducting nutritive particles to the mouth of oyster. The blood of oyster, called haemolymph is colorless (Figure 2).

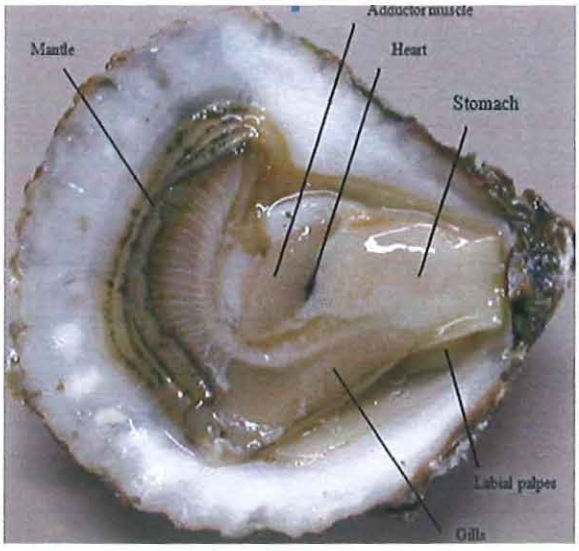


Figure 2: Ostrea edulis anatomy

I. 2 Haemocytes: immune system

Haemocytes can be compared to blood cells invertebrate. These cells circulate in oysters through a semi open circulatory system which contains haemolymph. Defence mechanisms of oyster rely on haemocyte cells. The main function of these cells is a phagocytic activity.

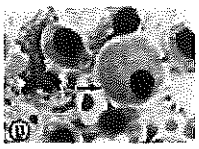
Haemolyphe can be found in various localizations in the circulatory system (vessels and sinus) but are also present in all tissues and bodies.

a) Morphology

Three types of haemocytes have been described in flat oyster *Ostrea edulis*: granulocytes and two types of hyalinocytes (large and small) [Chagot, 1989; Auffret 1989]. These cells are differentiated by differential morphological criteria such as: size, form, nucleo-cytoplasmic ratio and presence of cell organelles.

❖ Granular haemocytes also called granulocytes small nucleus and an abundant cytoplasm characterized by many granulations. . The nucleo-cytoplasmic ratio is weak. (figure 3)

Figure 3: Granular haemocytes



❖ Agranular cells haemocytes also called hyalinocytes: with a nucleo-cytoplasmic ratio more impotant compred to granulocytes. They are divided into two categories: according to their size the large and the small ones.(figure 4)

Figure 4: Agranular cells

The parasite Bonamia ostreae can be observed is present in the three haemocytic types but essentially in hyalynocytes[Cochennec *et al.*,2003].

b) Haemocyte functions

Haemocytes are the effector cells of bivalve immunity but they do not only have this function. They are involved in various processes such as nutrition, detoxification, wound repairs, digestion and transport of nutrients. The process of defense is primarily the phagocytosis. The phagocytosis of a

foreign body (bacteria, protozoan, protein or virus) can be describe into four principal stages (figure 5) [Auffret 1985; Cheng 1981; Fisher 1986]

1. Recognition of foreign particles: the meeting between hemocytes and particles can occur passively or actively by chemotactism. This recognition is done thanks to specific receptors and lectins.
2. Formation of phagosomes: the internalization of particles is carried out by the formation of phagosomes, this formation results from a rolling up of the external membrane of the haemocyte.
3. Internalization: Phagosomes fuse with lysosome in order to form a phagolysosome. During this formation, the lysosome releases lysosomal enzymes in the phagosome.
4. Destruction of the particles and release of the particles: Destruction of ingested particles is done through 2 mechanisms: (1) An oxygen independent one which involves lysosomal enzymes like hydrolase and (2) an oxygen depent one which relies on the production of reactive oxygenated species (ROS). Degradated particles are then released by exocytosis.

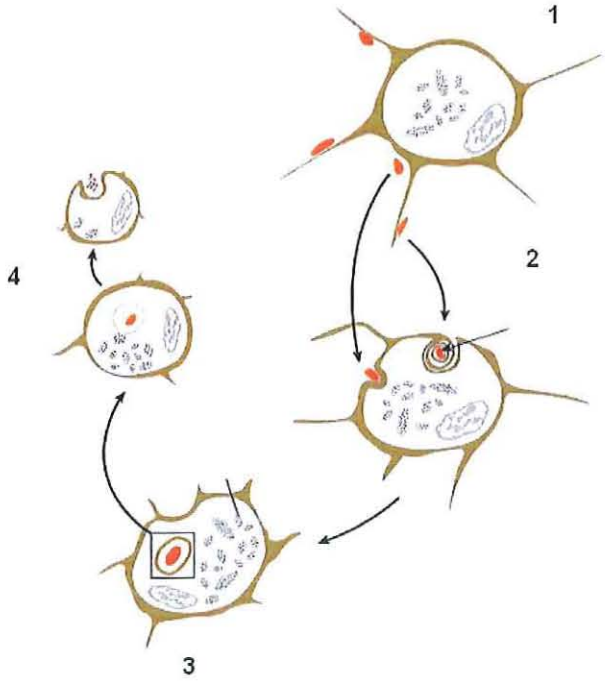


Figure 5: Diagram of phagocytosis is 4 stages
(according Xue 1998)

I. 3 The parasite: *Bonamia ostreae*

a) Context

In 1979, massive mortalities were observed in the flat oysters of the island Tudy in Brittany [Pichot 1979]. A still unknown parasite on the French coasts is found in the contaminated haemocytes of oysters. It is also known under the name of haemocytic disease of the flat oyster. This parasite belongs to the group of the microcells. The term of microcells applies at organizations, of small size (about some µm), usually rounds and has a circular core. This group is composed of very pathogenic organizations which induce strong mortalities among their respective hosts. Bonamiosis due to *Bonamia ostreae* is a

notifiable disease because of the economic impact this diseases has on the production of flat oyster. It should be noted that it is not about a zoonosis, the consumption of the flat oyster infected is not harmful for the man [Pichot *et al.;* 1979]

It is in 1963, on the Wests coast of North America, in particular in California that the parasite was hypothetically appear for the first time. The introduction of the parasite in France results from the importation of spawns oysters of the United States considered then as unscathed of all diseases. [Gizel 1985, Balonel 1983]

In France, *Bonamia ostreae* is present all along French coasts except in the North Coast, from right bank of the Seine to the Belgian border. *Appendix 4.*

Today the bonamiosis due to *Bonamia ostreae* is reported in Europe, North America and in Morocco.

b) Taxonomy and Morphology

Organisms of the group of microcells, to which *Bonamia ostreae* belongs, cannot be characterized by the lesions they induce, nor by their host.

Parasites of the *Bonamia* genus ultrastructural share same commune characteristics with parasites of the *Haplosporidium* genus, in particular the presence of dense bodies called haplosporosomes which support the inclusion of *Bonamia ostrea* within *Haplosporidum*. [Cochennec 2000, Pichot *et al. ;* 1979] *Appendix 5.*

This hypothesis was confirmed by the sequencing of 18S RNA. This study show than *Bonamia ostreae* belong to Haplosporidia.[Cochennec *et al.*, 2000]

The parasite is generally intracellular. *Bonamia ostreae* appeared like small cells from 2 to 4 µm at inside the cytoplasm of the haemocytes (Figure 6,7). It is present at the same time at the seed oyster and the adult. However, the death of the host generally intervenes as from two years; the intensity of the infection increases with the age and the size of oyster. [Balouet G. *et al.*, 1983, Grizel 1958, Caceres-Martinez 1995]

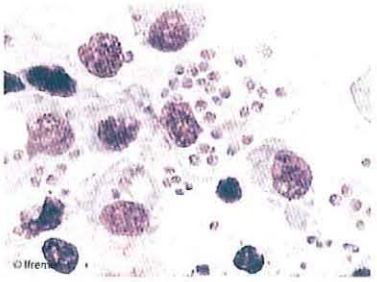


Figure 6: Optic microscope : *Bonamia ostreae* intrahemocytic (*x* 100). Apposition cardiac.

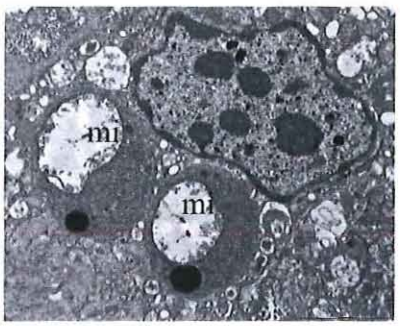


Figure 7: Electronic microscope: *Bonamia ostreae* intrahemocytic (mi: mitochondria).

The observation by electron microscopy makes it possible to distinguish two forms of parasite. [Pichot and Al 1979]

The dense form, which is most frequent in infected oyster fabrics. It has a core, ribosome, mitochondria, dense bodies structured such called haplosporosomes.

The clear form as for it presents some differences. The core can have a bulky nucleolus which is generally localized in periphery. The mitochondria have clearer and more peaks.

During advanced infections the parasite can be observed in extracellular position. Moreover, the infection is often associated an accumulation of hemocytes on the level of fabrics reached.

c) Parasite life and transmission mechanisms

Today, the complete cycle of *Bonamia ostreae* is not still elucidate. Only assumptions were elaborate. The site of entry of the parasite would be the gills. Indeed, these last are continuously in contact with sea water which can contain parasites.

Although the cycle of life apart from the host is not known, it was possible to transmit in experiments the disease to the laboratory by cohabitation or inoculation of purified parasites.

d) Clinical methods

Bonamiosis is a lethal infection of the haemocytes flat oysters, sometimes associated with by a yellow discoloration and extensive lesions located on the gills and the mantle. However, the majority of infected oysters have a normal appearance. At the histological level, the lesions occur in connective tissue of the gills, the mantle and the digestive system. These interhaemocytic protozoans invade the whole of the body quickly, and multiply to reach a considerable number of parasites, phase which coincides with the death of oysters.

The examination of molluscs infected by *Bonamia ostreae* shows in the majority of the cases a normally pigmented digestive gland and the presence of products of reserve. However, sometimes oysters show a greyish color and a nauseous smell characteristic of a pre-mortem phase. In addition, some lesions can be noted on one or more gill layers. If the presence of these lesions is very often associated with the parasite *Bonamia ostrea.* The disease can affect an oyster without generate these clinical signs. There is a correlation between the evolution of the disease and the lesions of the gills, the lesions being more important in strongly infected oysters. Lesions in gills appear like large perforations, located in the medium of the filament gill or at its base, and of indentations located on the level of the edge of the gills (Figure 8).

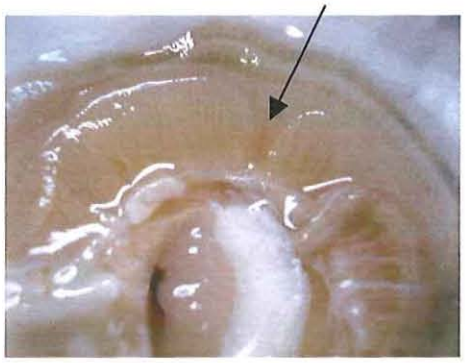


Figure 8 : Lesion of gills at *Ostrea edulis*

I. 4 Interaction between flat oyster and *Bonamia ostreae*

Bonamia ostreae life cycle is not completely known. The parasite would be introduced into oyster when this one filters sea water. The parasite would then take for targets the haemocytes which are the cells of the system of defense. Parasites adhere to haemocytes and are phagocytosed, by invagination of haemocyte plasmic membrane. Parasites survive within haemocytes in parasitophorous vacuole. The parasite cans growth without to be degraded.

The development of the protocol of purification of the parasite [Mialhe *et Al.*, 1988] and of haemolymph sampling allowed to study *in vitro* from highly infected oysters the interactions between haemocytes and parasites. Experiments consisting in contact between the parasite *Bonamia ostreae* and the haemocytes were carried out for two oyster species: *Ostrea edulis* and *Crassostrea gigas*. The results showed that the parasite is phagocytosed in both cases. However, if haemocytes of *Crassostrea gigas* degrade the parasite, this one persists in *Ostrea edulis* haemocytes.[Chagot 1992]. This underlines the interest of studying post-phagocytic phenomena. This study also reported a decrease of the internalisation of the parasite in the presence of Cytochalasine B for the both species. This observation indicates a possible implication of the parasite in its own internalisation.

In addition, it was shown that haemocytes treated with simple sugars (mannose, glucose, fucose, N-acetyl glucosamine and galactosamine), preserved their capacity of phagocytosis for the both oysters. Lectin type receptors thus do not seem involved in the internalisation of parasite. On the other hand, the treatment of the parasite with sugars before contact a decrease in the rate of infection for the haemocytes of the both species. These results suggest that the parasite would have some lectin type molecules on the surface, implied in the recognition by the haemocytes [Chagot 1989].

The cellular enzymatic characterization using APIZYM gallery showed significant differences between haemocytes from *Crassostrea gigas* and *Ostrea edulis*. The comparison of the post-phagocytic lysosomal activity showed a higher level in the haemolymph of cupped oyster compared to haemolymph of the flat oyster. [Xue 1998]

Moreover the distribution of the activities between the total haemolymph, the acellular fraction and haemocytes are different between the both species. Contrary to cupped oyster, the activities are more important in the total haemolymph and the acellular fraction of the flat oyster [Xue, 1998]. The enzymatic concentration of *Crassostrea gigas* haemocytes would thus make it possible to destroy the parasite after phagocytosis and thus to avoid the installation of the parasite within haemocytes [Naciri and Al, 1998]. The selected oysters would have less large agranular cells. The author suggested that the large agranular cells could have an important role in the development of the disease. [Cochennec 2000, Cochennec *et al.*, 2003]

Bonamia ostreae seems to induce a reduction in the number of granulocyte. This study carried out on healthy oysters and infected oysters, shows that the healthy oysters present granulocytes mainly, whereas the parasitized oysters present as a majority of large the hyalinocytes. This study also related to the effect of the parasite on the haemocytic activities. It thus seems that the parasite inhibits some haemocytic activities what probably enables him to be maintained in a cellular type without being degraded.

Thanks to work already completed and knowledge that we acquired, it is importance to make a transcriptomic study in order to better understand interactions between *Bonamia ostreae* and flat oyster.

Context of internship

It seems necessary to point out the work carried out by B. Morga before the beginning of my training course. A transcriptomic approach was carried out. B.Morga, identified genes of interests thanks to the technique of Subtractive Suppressive Hybridization (SSH), during an in vitro infection between haemocytes only on the one hand and the haemocytes put in presences of parasites on the other hand. The identified genes have various roles in the stages of phagocytosis. They can be classified in seven categories. *Appendix 6.* For example, it seems clear that the genes entering in plays at the time of the stage of the attachment are genes having a role on the membrane receivers and the cytoskeleton.

Moreover, B.Morga also identified two reference genes by RACE PCR (Rapid Amplification off cDNA ends): the elongation 1alpha gene and GAPDH gene. These two genes of household or reference, are genes whose expression does not vary or very little. After having selected the better of two genes, that will serve to us as "bench mark" to compare the variation of the form of genes of interests.

This work completed with the folding screen is the base of our study, thanks to that and with the technique of quantitative PCR, we will be able to determine the level of form of genes selected by SSH in an in vitro system between circulating oyster punts cells (haemocytes) and the parasite *Bonamia ostreae.* Indeed, the quantitative PCR is a tool of diagnostic interesting. It has sensitivity generally higher than the simple PCR and as its name indicates it, it makes it possible to quantify the required target.

In this context, our study related first of all to the standardization of the quantitative PCR. To conclude this study, it was necessary initially to draw specific starters of genes retained by SSH. After having selected the couples of starters and their most effective concentrations in quantitative PCR, we could obtain curves standards. For that the preparation of the samples required the plasmidic extraction of DNA.

Thereafter, the study of the chemistry of the quantitative PCR and the choice of a gene of reference enabled us to show under expression as well as up-expression of certain genes at the time of the setting in contact of the parasite.

II. Materials and Methods

II. 1 Biological materials

a) Animals

Flat oysters *Ostrea edulis* used during this study came from Bay of Quiberon (Southern Brittany). It's an endemic zone for the bonamiosis. The oysters were two years old. These oysters were stored in raceways of 120 liters. The sea water was enriched in phytoplankton (*Skeletonema costatum, Isocchrisis galbana, Chatoceros gracialis and Tetraselmis succica*).

b) Haemolymph collection

Haemolymph was withdrawn from the adductor muscle of 20 flat oysters, a 1mL syringe equipped with a needle (0.40x90) was used. Haemolymph samples 10 ml were filtered on a 60 µm mesh to eliminate some tissue, macroparticles and maintened on ice. Haemolymph samples were pooled, and haemocyte counting were performed using a Malassey cell and the cell concentration was adjusted at 2.10^6 cells ml^{-1} with 0.22 filtered salty sea water (FSSW) at 0.22µm (FSSW).

c) *Bonamia ostreae* parasites

Bonamia ostreae was purified according to the protocol of Mialhe et al. (1988). Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After homogenization of all the organs except the adductor muscle, the parasites were concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient. Finally, the purified parasites were resuspended in FSSW (0.22 pm). *Bonamia ostreae* cells were then counted using a Malassez-cell. Appendix 7

d) Clones

A collection of clones is stored at –80°C. These clones are put in suspension culture in Luria-Bertani (LB) Ampicilin during overnight at 37°C for obtain bacteria with the interested fragment contain in the plasmid PCR2 TOPO.

II. 2 Methods

a) In vitro infection

Infected cells were obtained in vitro by putting in contact haemocytes with purified parasites during 12 hours in flasks. For that purpose, 5 ml of haemocytes at a concentration of 2.10^6 /ml were introduced in flak and maintained during 2 hours until the formation of cells lawn supernatant was then withdrawn and replaced by the parasite suspension on FSSW during 2 hours. After 2 hours, supernatant previously filtered at 0.22µm to eliminate bacteria was introduced again in flasks and cells were then incubated during 12 hours at 15°C. After 12hours of incubated cells were rinsed twice with phosphor buffer saline (PBS 1X).

b) plasmidic DNA extraction

Plasmidic DNA extraction was extracted from bacterial cultures which had grown 1 night at 37°C. Plasmidic DNA extraction was performed using the Flast Plasmid minikit (Eppendorf) according to the Manufacture procedure. *Appendix* 8. Finely, plasmidic DNA was linearized by digestion with the *XhoI* enzyme.

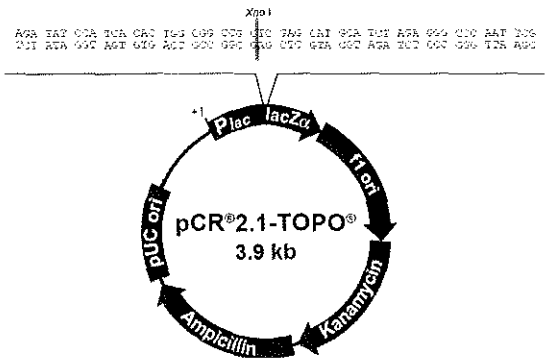


Fig 9: Plasmid PCR 2 TOPO

c) RNA Extraction

The total RNAs were extracted from the haemolymph pools using Trizol® according to the protocol provided by the manufacturer invitrogen and appended at the end of this report *Appendix 9.*

After PBS 1X rinsing, cells were lysed and recovered by addition of 1 ml Trizol®. RNA precipitation was carried out by addition of cold isopropanol (660 µl). RNA quality and quantity were determined by spectrophotometry. RNA suspensions were adjusted for quantity of 1.5µg/ml

d) RTranscriptase

The reverse transcriptase is an enzymatic reaction allowing the synthesis of complementary DNAs from messengers RNAs. Prior to this reaction, DNA present in the samples are destroyed, it is necessary to destroy the DNA by adding Rnase-Free Dnase for 45 min at 37°C. The digestion is finaly blocked by adding the enzyme Dnase stop during 10min at 65°C.

e) Real time QPCR

The polymerase chain reaction (PCR) is an amplification reaction of a specific part of a given nucleic acid (DNA). PCR allows an exponential amplification of nucleic acid sequence by using two primers. These primers (F and R) bind to its complementary sequence.

Polymerase stars at each primer and copy the sequence of that strand. At each cycle, double stranded molecules of both the original DNA and the copies are separated (denaturation step) primers bind again to complementary sequences (annealing step) and the polymerase replicates them (extension step).

In quantitative PCR the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule, in our study is SYBR GREEN II (agent $C_{32}H_{37}N_4S$ aromatic Appendix 11). The point at which the fluorescence signal is measured in order to calculate the initial template quantity can be at the end of the reaction or in our case while the amplification is still progressing (Real Time QPCR)

The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration, with QPCR instrument systems collecting data for each sample during each PCR cycle. The first cycle at which the instrument can distinguish the amplification generated fluorescence as being above the ambient background signal is called the "Ct" or threshold cycle. This Ct value can be directly correlated to the starting target concentration for the sample. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample (Figure 10). The Mx3000P analysis software determines the Ct value for each sample, based on certain user-defined parameters. If a standard curve dilution series has been run on the same plate as the unknown samples, the software will compare the Ct values of the unknown samples to the standard curve to determine the starting concentration of each unknown. Alternatively, the software can use the Ct values to generate relative comparisons of the change in template concentration among different samples.

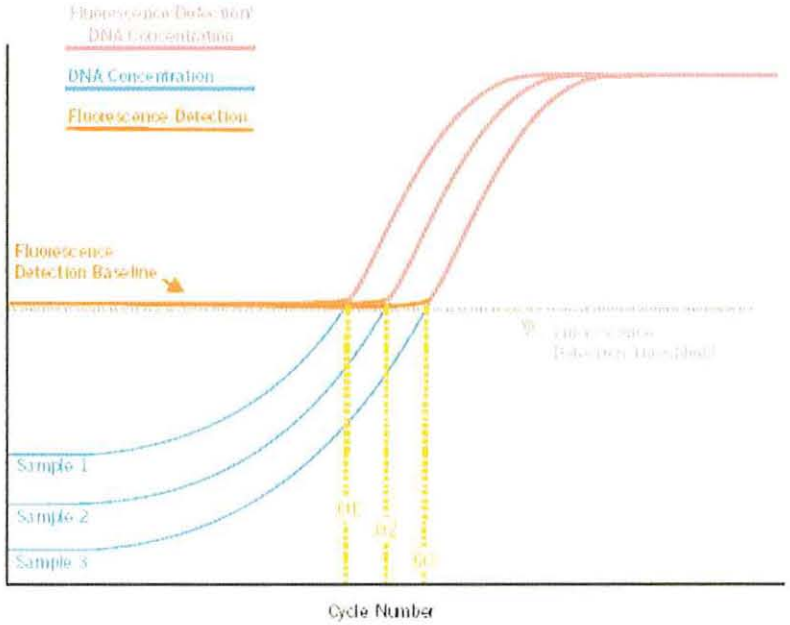


Figure 10: Principles of real-time fluorescence detection and QPCR target concentration measurements using threshold cycle (Ct). The Ct is inversely proportional to the initial copy number. Only when the DNA concentration has reached the fluorescence detection threshold can the concentration be reliably inferred from the fluorescence intensity. A higher initial copy number will correlate to a threshold cycle.

PCR analyses were performed using for each sample:

-2.5 µl of Forward primer (table 2)

-2.5 µl of Reverse primer (table 2)

-2.5 µl of water

-12.5 µl of Mix Sybr GreenII

After that, 5 µl of cDNA at 2.10^{-2} or of plasmidic DNA were added to the 20µl of PCR Mix. We have work in triplicates. The software calculates the average of three samples. The number of cycle choice was of 40 and the profile thermique was of 60°C for hybridation primers. Appendix 10.

Results are presented here as changes in relative expression normalized to the reference gene (EF1), determined using the equation:

$$\text{Relative expression} = [(E_{target})^{\Delta Cttarget(control-sample)}] / [(E_{ref})^{\Delta Ctref(control-sample)}]$$

where E_{target} is the amplification efficiency of the target or gene of interest, E_{ref} is the amplification efficiency of the reference (EF1) and Ct is the crossing threshold.

Table	Name	Sequence
1	ElongPCRQ5m	Forward: GTCGCTCACAGAAGCTGTACC Reverse: CCAGGGTGGTTCAAGATGAT
2	GAPDHHG	Forward: TCCCGCTAGCATTCCTTG Reverse: TTGGCGCCTCCTTTCATA
3	cytoxy3	Forward: GGGGCCAGCTATAACATTCA Reverse: GATCACGGGTTCACCCATAC
4	OGST2	Forward: GGTCGTCAGGGGTCAGTTT Reverse: GGTTCCCGTTCTTGAGCA
5	CLAT	Forward: TCTTACTCGTCTCCTGGACCTC Reverse: CGGAAGTGTGTGTGAGTTGG
6	SRC	Forward: TGGTGTTGATAACCCGACAA Reverse: TCGGGCGTCATAATCATACA
7	CYTOP450	Forward: GTCATCAAGCGAATGCGATA Reverse: GGAGAGCTCCCTCATTTTCC
8	TETRA	Forward: TTCCATCCATTGCTGATTTG Reverse: AGCTGAACTCTGCCGTGAAG
9	ARP	Forward: TGCTCGTTGCATGAGGAA Reverse: ATTCGGTGCGCATTTCA
10	TIMP	Forward: TTCGACGCATGTATGAAAGG Reverse: TCTGGATTCAGGCCCCTA
11	gal	Forward: TCGGAGGTCGCCCTTAAT Reverse: TTGCCGTGAACAATCAACA
12	Hsp9	Forward: TTTGTGGAACGGGTCAAAA Reverse: AACGTCGAGCACAGTCGAG
13	SOD	Forward: TCGTCAATGTCAGCGTGAA Reverse: AAATGTTGGGGCTGGTGA
14	Filamin	Forward: TGATTTAACCGACGGGAAAG Reverse: CTTCATCACCGGTTTGTGG
15	Lipo	Forward: GAATGGTCTGGGGAACCA Reverse: TTTCCATTCCACGCACAA
16	WD40	Forward: AGCGCCATTTTCTTTGTCA Reverse: ACACAGCCCAGTCACTTCAA

Table 2: Sequences of the forward and reverse primers used for real time qPCR

f) Sequencing and sequence analyse

PCR products were purified using Montage® Centrifugal Filter Devices according to the Millipore Kit. 100 µL of PCR reaction was addict into the sample reservoir, the sample reservoir contain 300 µL distilled water.

Montage PCR centrifugal filter devices are disposable, single-use centrifugal devices for processing aqueous biological solutions in the 0.1 to 0.5 ml volume range. They are used in fixed angle micro-centrifuge rotors that accommodate 1.5 ml microfuge tubes. Used for PCR product purification, Montage PCR devices allow for up to 500 µl sample clean-up of salts and primers with a concentration factor of 5× (typical PCR in 100 µl) in 15 minutes, with no solvents or chemicals required. Montage PCR devices achieve high recoveries of fully functional nucleic acids. The Montage PCR device consists of a filtrate collection vial with attached cap and a sample reservoir. The sample is spun to "virtual" dryness in a 15-minute spin time, followed by sample reconstitution, and an invert spin transfer into a clean vial for subsequent analysis and/or storage.

The sequencing reaction was carried out into a 10 µl final volume, containing 6.3 µl purified PCR products,1.8 µl of sequencing buffer, 0.4 µl of BigDye® Terminator v3.1 (Applied Biosystems), 1.5 µl of primer F or R at 5µM of concentration initial (primers same F or R used for the PCR). The programme consisted in initial denaturation of 3 minutes at 96°C followed by 35 cycles of 30 s at 96°C, 30s at 55°C and 4 minutes at 60°C. This reaction was performed using a 96-well plate. Sequencing reactions were then purified. In each sample, 60 µl of 100% ethanol were added and samples were centrifuged at 3000 rcf for 30 minutes. The plate was inverted to remove ethanol. Then, 60 µl 70% ethanol was added, followed by a centrifugation of the plate at 1650 rcf for 10 minutes. The plate was then centrifuged upside down for 30s to remove the ethanol. The elimination of ethanol is important because residuals could induce artefactual peaks when running samples onto the sequencing machine. Finally samples were dried in a Speed Vac and re-suspended with 10 µl formamide.

Samples were loaded into an ABI PRISM® 3130 XL-Avant Genetic Analyzer, using a 36 cm capillary array and POP 7 polymer.

g) Statistical analysis

These statistical analyses were performed using the triplicate realtime PCR assay values obtained for each sample; the graphs (Figure. 15) present the mean values with standard deviations.

III. Résultats

III. 1 RT-QPCR standardization

a) Determination of primer concentration

Ranges of dilution were prepared from plasmidic DNA and tested by RT QPCR using different primer concentration (see table 3). Optimum primer concentrations were determined by taking into account the efficacy of the reaction and by checking the absence of cross breeding between primers. The best combination allowed obtaining a minimum Ct value. This work has been down in triplicate and for the 14 primer pairs. For example, for the amplification of the fragment OGST, the minimum Ct value that could be obtained with an efficacy of 100% was with primers concentrations of 3µM (see table 3)

		Reverse		
Foward	Ct's	**0.75 µM**	**1.5 µM**	**3 µM**
	0.75 µM	17,33	15,25	14,43
	1.5 µM	16,72	14,61	13,95
	3 µM	16,26	14,76	13,84

Table 3: Ct value obtain by Q PCR in function of various primer concentration Forward and Reverse.

Lack of primer dimer and specificity of amplified products were appreciated by looking at the dissociation curve. More specifically by checking the absence of amplification in negative samples (NTC) and the presence of a unique peek at specific melting temperature. For example, Figure 11 shows an expected dissociation curve (no amplification in NTC, unique peek of amplification). Figure 12 shows dissociation curve for which primer dimmers were observed.

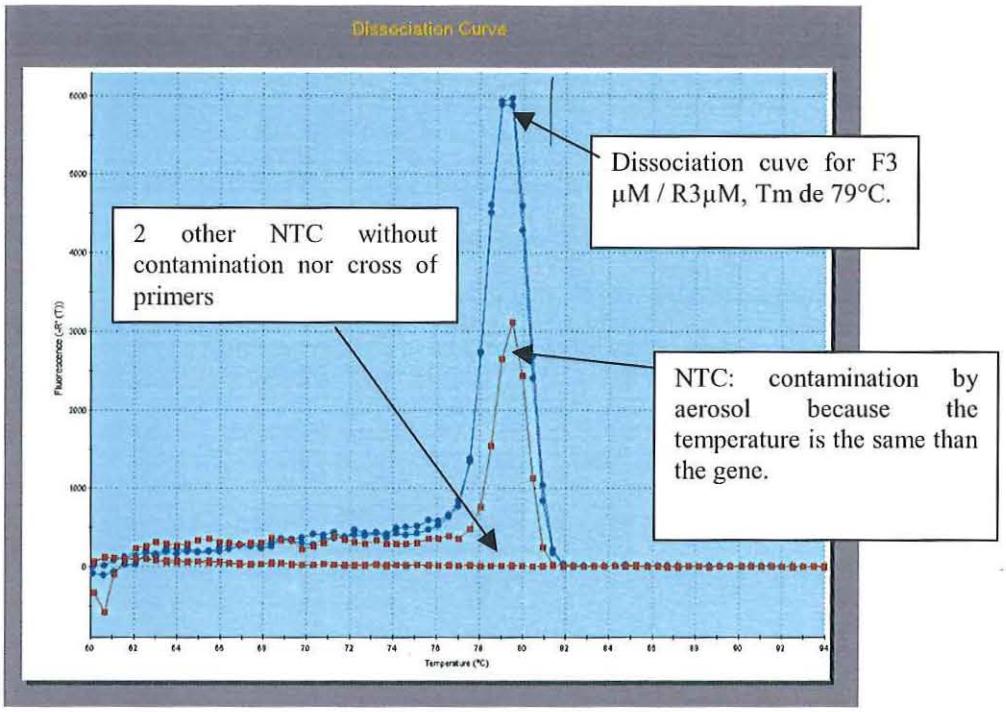


Figure 11 : Dissociation curve shows the fluorescence in function of the specific melting temperature (Tm). The blue peek is the Tm of specific primer and red curve are NTC.

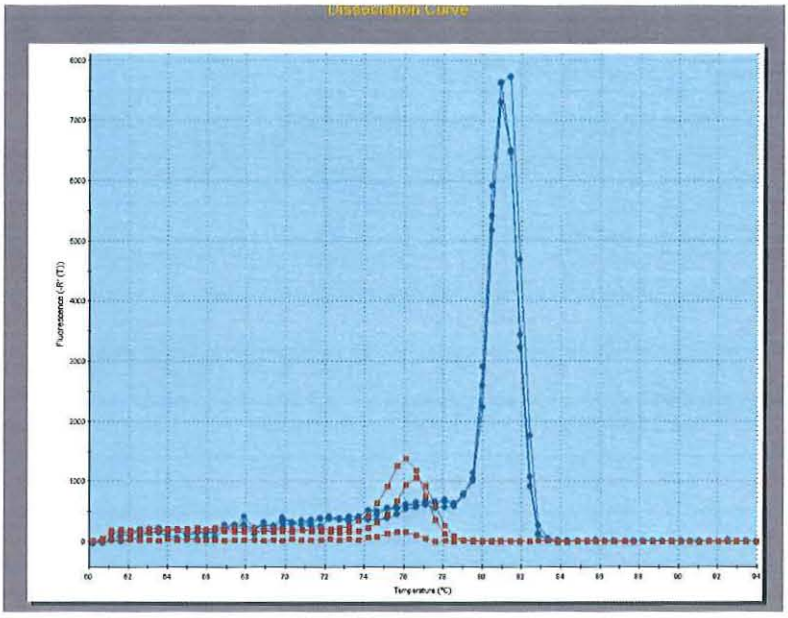


Figure 12: Dissociation curve shows the fluorescence in function of the specific melting temperature (Tm). The blue peek is the Tm of specific primer and red curve are NTC shows primer dimer.

b) Standard cuves

After the optimization in primers, standard curves have been realized for the 15 selected genes using linearized plasmidic DNA (digested by XhoI).

For that purpose, dilution ranges of plamidic DNA were prepared from 2µg/ml to 2.10-6 µg/ml. Each dilution was tested in triplicate. Figure 13 shows the standard curve obtained for the gene OGST and allows to appreciate homogeneity between triplicates and efficacy of the reaction. In addition, standard cuve allows to check the accuracy of the dilutions of 10 to 10, the difference between two Ct or ΔCt

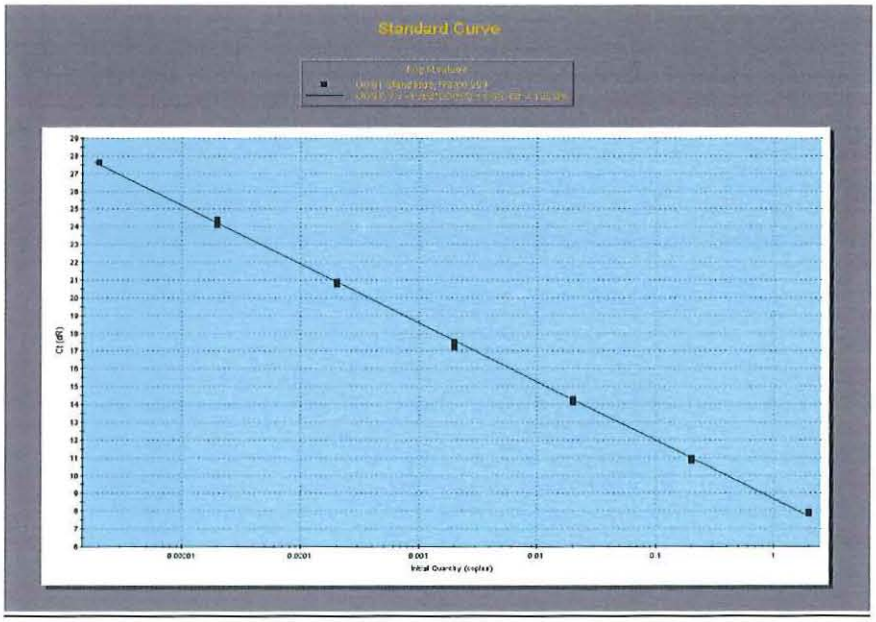


Figure 13: Standard curve various Ct value in function of 7 dilutions DNA plasmidic from OGST

This experience was performed for the selected genes and results are synthetized in table 4.

Gene	Name amorce	Concentration F (µM)	Concentration R (µM)	Efficacity (%)
Cytochrome oxydase III (OXY3)	cytoxyIII	3	3	100.5
Elongation EF-1 (Elong)	Elong (5m)	3	3	99.7
Cytochrome P450 (P450)	P450	3	3	99.8
Actin regulator polymerisation 2/3 (ARP)	Actin	1.5	3	100.6
receptor LIPO	Lipo	3	3	99
Heat Stock Protein90 (HSP)	HSP9	3	3	99.9
Galactin	GAL	3	3	101.7
Tetraspanin (TETRA)	TETRA	3	3	103.9
Mito antigen protein kinease (MAPK1)	WD40	3	3	100.5
Filamin	Filamin	3	3	101.4
Oméga gluthatione s transférase (OGST)	OGST2	3	3	100.8

Tissu Inhibitor of MetalloProtease (TIMP)	TIMP	1.5	3	107.3
Super Oxyde Dismutase (SOD)	SOD	3	3	106.3
Clathrin	Clathrin	3	3	99.4
SRC	SRC	3	3	104.4

Table 4: results of selected primer, their concentration R and Fn and their efficacy.

III. 2 Expression of genes during a *in vitro* infection between the parasite *Bonamia ostreae* and haemocytes of flat oyster

After the standardization of real time QPCR, the expression of various genes was studied in haemocytes of flat oyster put in contact with *Bonamia ostreae* and haemocytes alone. Total RNA was extracted from a pool of haemocytes infected and from a pool of haemocytes alone 9.5.µg/ml and 11.4 µg/ml of RNA could be obtained respectively. The quality of the extracted RNA was appreciated on an agarose gen as shows by figure 14.

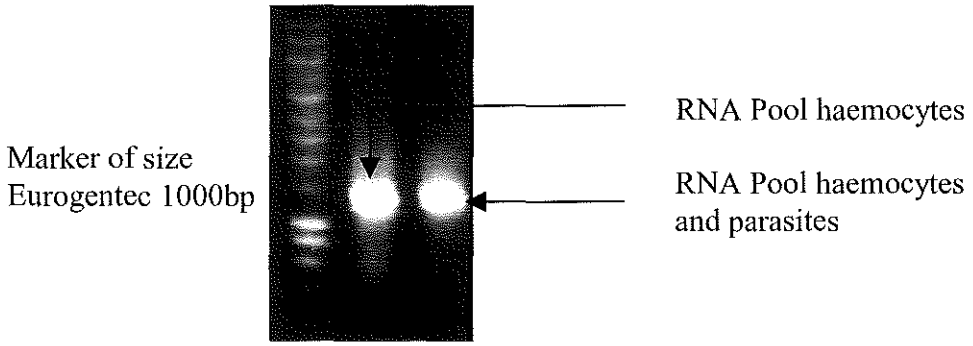


Figure 14 : Control of RNA extraction by agarose gel 1.2%

Expression of selected genes was measured by comparison with a reference gene called too house keeping gene. That is a gene for which expression is stable in the different tested condition.

The expression of the elongation factor gene EF-1 measured in haemocytes close and in infected haemocytes was equivalent. The EF-1 gene was thus used as a normalizer for the measure of the relative expression of other gene. Results are presented in figure 15.

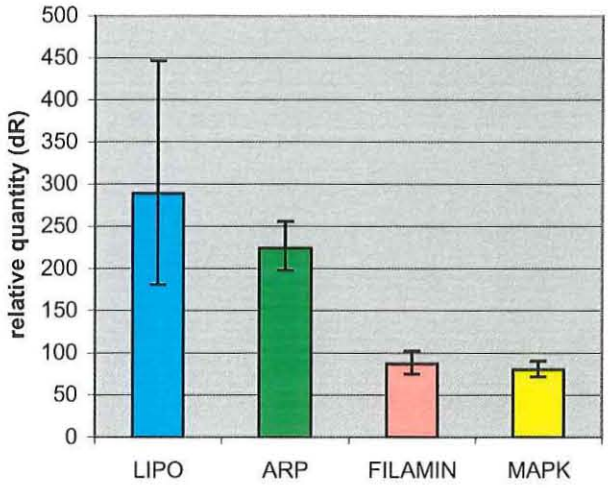


Figure 15: Results from real-time RT-PCR showing temporal expression patterns of some representative cDNA transcripts over a 12 hours of contact haemocytes and parasite. Relative expression levels were normalized to EF1. Bars represent the mean of three replicates per sampling point and the error bars correspond to the SD.

ARP, Filamin Lipo receptor, MAPK1 and OGST displayed increased transcript levels 230, 80, 90 and 2,5 fold higher than control respectively. (figure 15 and 16)

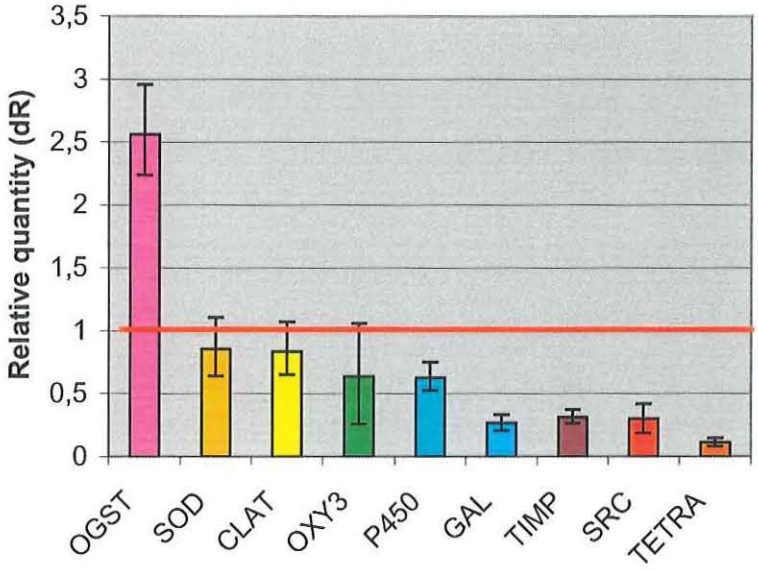


Figure 16: Results from real-time RT-PCR showing temporal expression patterns of some representative cDNA transcripts over a 12 hours of contact haemocytes and parasite. Relative expression levels were normalized to EF1. Bars represent the mean of three replicates per sampling point and the error bars correspond to the SD. Under the number 1, genes are up-regulated

On the contrary, transcript levels of SOD, clathrin, OXY3, Cytochrome P450, Galectin, TIMP, SRC and Tetraspanin are down- regulated respectively to 1.2 , 1.3 , 2.5, 2.5, 4, 3.5, 3.5 and 5 less than control. (figure 16)

All QPCR products were downloaded on an agarose gel in order to check the presence of one unique products and its size (100 and 150 bases pair) (see figure 17 for example). All PCR products were further sequenced in order to confirm their specificity. After treating sequences using the software CHROMAS PRO 1.49. They were aligned with the sequences obtained from the SSH approach using the software ALIGN two sequences in NCBI web sites. All obtained sequences showed 100% of homology in the original ones

Figure 17 :Control of cDNA agarose gel of EF-1, Cytochrome P450, ARP and MAPK1 after amplification by qPCR

IV. Discussion

The study of gene expression is frequently used to understand interactions between host and pathogen notably for human or pet parasites like *Giardia lamblia* [Roxstrom- Lindquist et al. 2005], *Trypanosoma congolense* [Hill et al. 2005], *Plasmodium falciparum* [Bozdech et al. 2003] or *Perkinsus marinus*, parasite of cupped oysters *Crassostrea virginica* and *C.gigas* [Tanguy et al. 2004].

We choose to use such approach to investigate interactions between flat oyster and parasite *Bonamia ostreae.*

This study is inkeeping with the general pattern of research developed in the laboratory to understand the mechanisms of resistance of flat oyster against infection with *Bonamia ostreae*. Thanks to work realized by B.Morga and notably the SSH, we had at our disposal a bank of clones of various studied genes. We have selected genes because they belong to gene family implicate in the phagocytosis like cytoskeleton , gene of stress, detoxification and cellular mechanisms.

These genes belong at various families: Cytochrome Oxydase III and Cytochrome P450 play a role in the breathing chain. Filamine, MAPK1 and ARP are essential to formation of cytoskeleton. Receptors Lipo and Galectin code for membrane receptor. Tetraspanin, SOD and OGST are genes involved in the detoxification process.

In a firt step, the real time quantitative PCR was standardized for each selected gene using plasmidic DNA from clones obtained though the SSH. The linearization of plasmid PCR2 TOPO allowed a best hybridation of primers. Indeed, we noticed that efficacy of the quantitative PCR was better when the plasmide was digested. This can be due to a better accessibility of DNA, the obstruction space is less important than when it is circular.

Standardization allowed to determine primer concentrations the should be used for gene expression measures. During our tests, only one couple of primers, those amplifying of Elogation faction EF-1 presented unexpected DNA dimers. By looking at the sequences of these primers, it appeared several possibilities of homologies between the forward and the reverse primer, suggesting potential hybridation between them. New primers were then designed and were standardized with success.

An important step of my work was also to validate a reference gene for the QPCR. This last gene was the elongation factor EF-1 which did not present fluctuation of expression between tested conditions. The use of several housekeeping genes are recommended in gene expression studies.[Nygard et al 2007; Gonzalez-Ibeas et al 2007]

In a second step, expression of selected genes during an *in vitro* infection was investigated. For that purpose haemocytes were put in contact with parasites during 12 hours. This time corresponds to the conditions used to perform the SSH realised by B.Morga.

12 hours allow to measure post phagocytosis reactions, 4 hours are necessary to produce mRNA. Thus 12 hours allow to measure expression of genes involved in reactions accuring shows 8 hours after contact between parasites and haemocytes.

Among studied genes, four appeared up-regulated in infected haemocytes compared to haemocytes alone: the lipid receptor involved in recognition and attachment of parasite, MAPK1 involved in actin activation; ARP in the actin polymerisation and Filamin, actin binding protein which plays a role in the formation of actin filaments.

These four genes are involved directly or indirectly in the cytoskeleton and also in the penetration of parasite. These results are in accord with previous studie. [Chagot 1989]. Indeed, the treatment of haemocytes or parasites using cytochalasin B induced a decrease of *Bonamia ostreae* phagocytosis. Indeed cytochalasine B prevents actin molecule polymerisation, and as a consequence, it inhibits phagocytosis. [Davies et Allison, 1978]. It is possible that parasite possess a penetration active.

Omega gluthation s transferase was also found a up-regulated but in lesser concern. The gene were implicated in cascade of detoxification. They are important in detoxification of many xenobiotics.[Guillou 2006].

Tissu Inhibitor of MetalloProtease (TIMP) is interesting because he plays a key role in the repair of the wounds and the defence mechanisms [Montagnani et Al, 2001]. Associated with this gene, the clathrin is also interesting because it is involed in the phagocytosis of pathogens [Veiga et Al, 2007]. The clathrin is a protein used during phagocytosis. Src protein kinase is a protein which is expressed during the tyrosin phosphorylation cascade and appears essential for the activation of phagocytosis [Suzuki et al. 2000]. .

8 genes appeared down regulated in infected haemocytes compared to haemocytes alone.

Some of these genes like cytochrome oxydase III, SOD and P45, certain are involved in the degradation of exogenous molecules It is thus possible that parasite inhibits the expression of these genes in order to not be degradated.

However, we can think that implication of other lectin like mannose, lactose or the fructose. Indeed, studies realized by D. Chagot suggest that lectins would play a role in the modification of the membrane before endocytosis.

Our work brings to new light on interactions between *Ostrea edulis* and the parasite *Bonamia ostreae* notably interactions during the post phagocytosis. The parasite seems to active its own phagocytosis and then to inhibit its degradation.

Conclusion and perspectives

The work done during this stage was realised in order to study the interactions obtained between host and parasite. More specifically, 14 genes of interest through a SSH approach were selected for a study of gene expression in context of an experimental *in vitro* infection between haemocytes and *Bonamia ostreae.*

It was first necessary to standardize the real-time PCR before studying the expression of genes.

Standardization aimed at checking efficiency and quality of amplicon generated with a pair of specific primers by gene. This standardization was carried out on all the genes of interest and the reference gene.

Measures of gene expression allowed to up regulated and some down regulated. The identification of up- regulated and down regulated genes expressed involved in the cytoskeleton and a membrane receptor shows great interest to study these parameters by other approaches.

To validate results of this study it is necessary to repeat *in vitro* infections. The use of oysters resistant to the disease would provide additional information to understand the development of disease and the mechanisms developed by the parasite to escape the degration .

In the present work, we tested two conditions infected and no infected haemocytes. The standardization of the QPCR assay and the first results we could obtained are promising for further studies like the evolution of gene expression according to the time of incubation between parasite and haemocytes.

Bibliographie

Anonymous (1): http://www.fao.org/fishery/culturedspecies/Ostrea_edulis_fr

-Auffret, M. (1985). "Morphologie comparative des types hémocytaires chez quelques mollusques bivalves d'intérêt commercial." *Thèse de 3ème cycle Université de Bretagne Occidentale: 153 p.*

-Auffret, M. (1989). "Comparative study of the haemocyte of two oyster species: The european flat oyster, *Ostrea edulis, Lineaeus*, 1750 and the Pacific oyster, Crassostrea gigas (Thumberg, 1793)." *Journal of Shellfish Research 8: 367-383.*

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

-Blanchet E., (2005), *thèse:* " La bonamiose de l'huître plate *Ostrea edulis*: bilan des connaissances actuelles, analyse des données collectées en France de 1989 à 2004, mesures de lutte et préventives . "

-Bozdech, Z., et al. (2003) "Epression profiling of the schizont and trophozoite stage of Plamodium falciparum with a long-oligonucleotide microarray". *genome Biology* 4, R.9

-Buestel, D. (2004). "Etat actuel de production des mollusques en France, le cas de l'ostréiculture." *IFREMER.*

-Caceres-Martinez, J., J. A. F. Robledo, et al. (1995). "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.* 130(2): pp. 15-23.

-Chagot, D. (1989). "Caractérisation morphologique et fonctionnelle des hémocytes *d'Ostrea edulis* et Crassostrea gigas, mollusques bivalves. Etude invitro de leurs interactions avec le protozoaire Bonamia ostreae (Ascetospora) ." *Thése du diplôme de l'école Pratique des Hautes Etudes de Montpellier: 70 pages.*

-Chagot, D., V. Boulo, et al. (1992). "Interactions between Bonamia ostreae (Protozoa: Ascetospora) and hemocytes of Ostrea edulis and Crassostrea gigas (Mollusca: Bivalvia): Entry mechanisms." Journal of Invertebrate Pathology [J. INVERTEBR. PATHOL.] 59(3): pp. 241-249.

-Cheng, T. C. (1981). "Invertebrate blood Cells." *Academic press of London: p 233-299.*

-Cochennec-Laureau, N., M. Auffret, et al. (2003). "Changes in circulating and tissue-infiltrating haemocyte parameters of European flat oysters, Ostrea edulis, naturally infected with *Bonamia ostreae." J Invertebr Pathol* 83(1): 23-30.

-Cochennec, N., et al. (2000) "Detection of Bonamia ostreae based on small subunit ribosomal probe." *Journal of Invertebrate Pathology* 76, 26-32

-Davies,P and Allison (1978) "effects of cytochalasin B on endocytosis and exocytosis. In cytochalasins: Biochemical and Cell-Biological Aspect pp143-160.

-Espiau, B. (1999). "Caractérisation des populations hémocytaires de l'huître plate *Ostrea edulis* à l'aide de lectines." *Rapport de stage de BTS: 36 p.*

-Fisher, S. W. (1986). "Structure and functions of oyster haemocytes." *Immunity invertebrates 3: p 25-35.*

-Gonzalez-Ibeas, D et al (2007) "Melogen: an EST database for melon functional genomics" *BMC Genomics.*

-Goulletquer, P and Heral, M (1997) « Marine Molluscan Production Trends in France : From Fisheries to Aquaculture.

-Grizel, H. (1985). "Etude des récentes épizooties de l'huître plate *Ostrea edulis* Linné et de leur impact sur l'ostréiculture bretonne." *Thèse doctorat d'Etat en Sciences Naturelles, Université de Monpellier.3*

-Guillou, F et al. (2006) « Identification and expression of gene transcripts generated during an anti-parasitic reponse in *Biomphalaria glabrata* » Science direct.

-Hill EW, O. G. G., Agaba M, Gibson JP, Hanotte O, Kemp SJ, Naessens J, Coussens PM, MacHugh DE (2005). "Understanding bovine trypanosomiasis and trypanotolerance: the promise of functional genomics." *Veterinary Immunology and Immunopathology* 105((3-4)): 247-58.

-Mialhe, E., E. Bachere, et al. (1988). "Isolation and purification of the protozoan Bonamia ostreae (Pichot et al. 1980), a parasite affecting the flat oyster Ostrea edulis" *L'Aquaculture 71(4): pp. 293-299.*

-Montagnani, C., F. Le Roux, et al. (2001). "Cg-TIMP, an inducible tissue inhibitor of metalloproteinase from the Pacific oyster Crassostrea gigas with a potential role in wound healing and defense mechanisms(1)." *FEBS Lett* 500(1-2): 64-70.

-NaciriGraven, Y., A. G. Martin, et al. (1998). "Selecting the flat oyster Ostrea edulis (L.) for survival when infected with the parasite Bonamia ostreae." *Journal of Experimental Marine Biology and Ecology 224(1): 91-107.*

-Nygard, A. et al (2007) "Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR". *BMC molecular Biology*

-Pichot (Y.), Comps (M.), Tige (G.), Grizel (H.) et Rabouin (M.-A.), (1979). "Recherche sur Bonamia ostreae gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis*"

-Roxtrom-Lindquist, K., et al. (2005) Giardia immunity an update. Trends in parasitology 22, 26-31

-Suzuki, T., H. Kono, et al. (2000). "Differential involvement of Src family kinases in Fc gamma receptor-mediated phagocytosis." J Immunol 165(1): 473-82.

-Tanguy, A., et al. (2004) "Discovery of genes expressed in reponse to perkinsus marinus callenge in Eastern (Crassostrea virginica) and Pacific oyster (C.gigas)". Gene 338, 21-31

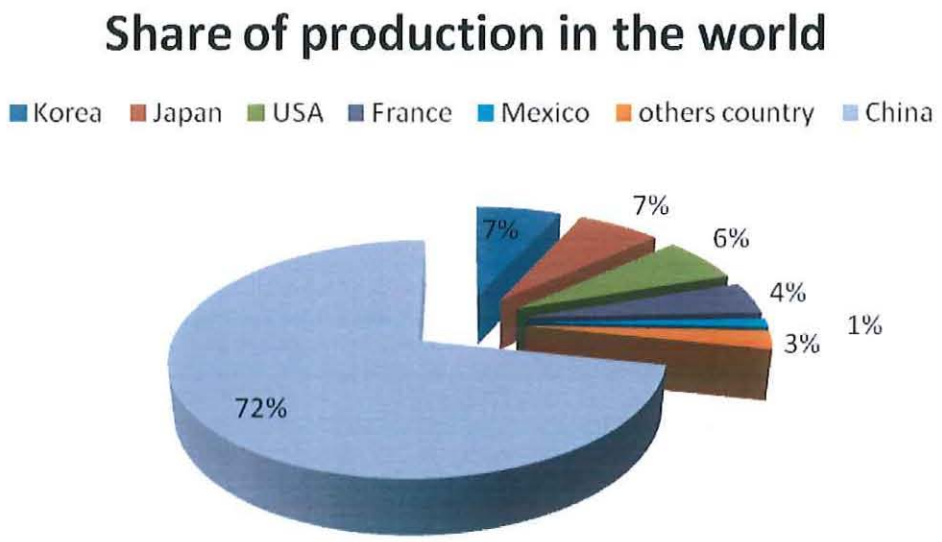
-Veiga, E., J. A. Guttman, et al. (2007). "Invasive and adherent bacterial pathogens co-Opt host clathrin for infection." Cell Host Microbe 2(5): 340-51.

-Xue, Q. (1998). "Caractérisation morphologique et fonctionnelle des hémocytes chez l'huître plate, Ostrea edulis." *Thése de Doctorat, Université de Bretagne-Occidentale Brest*: 324 pages.

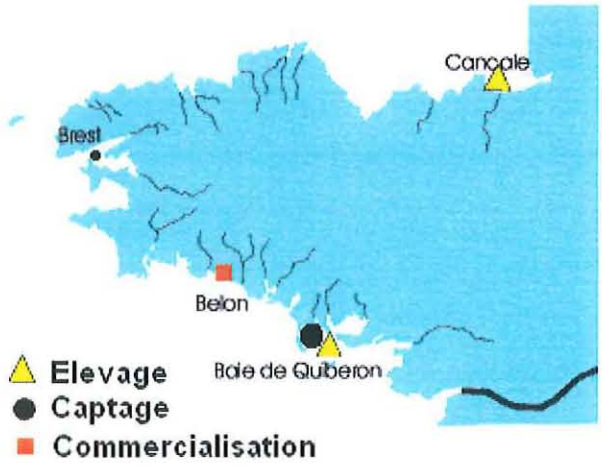
APPENDIX

APPENDIX 1

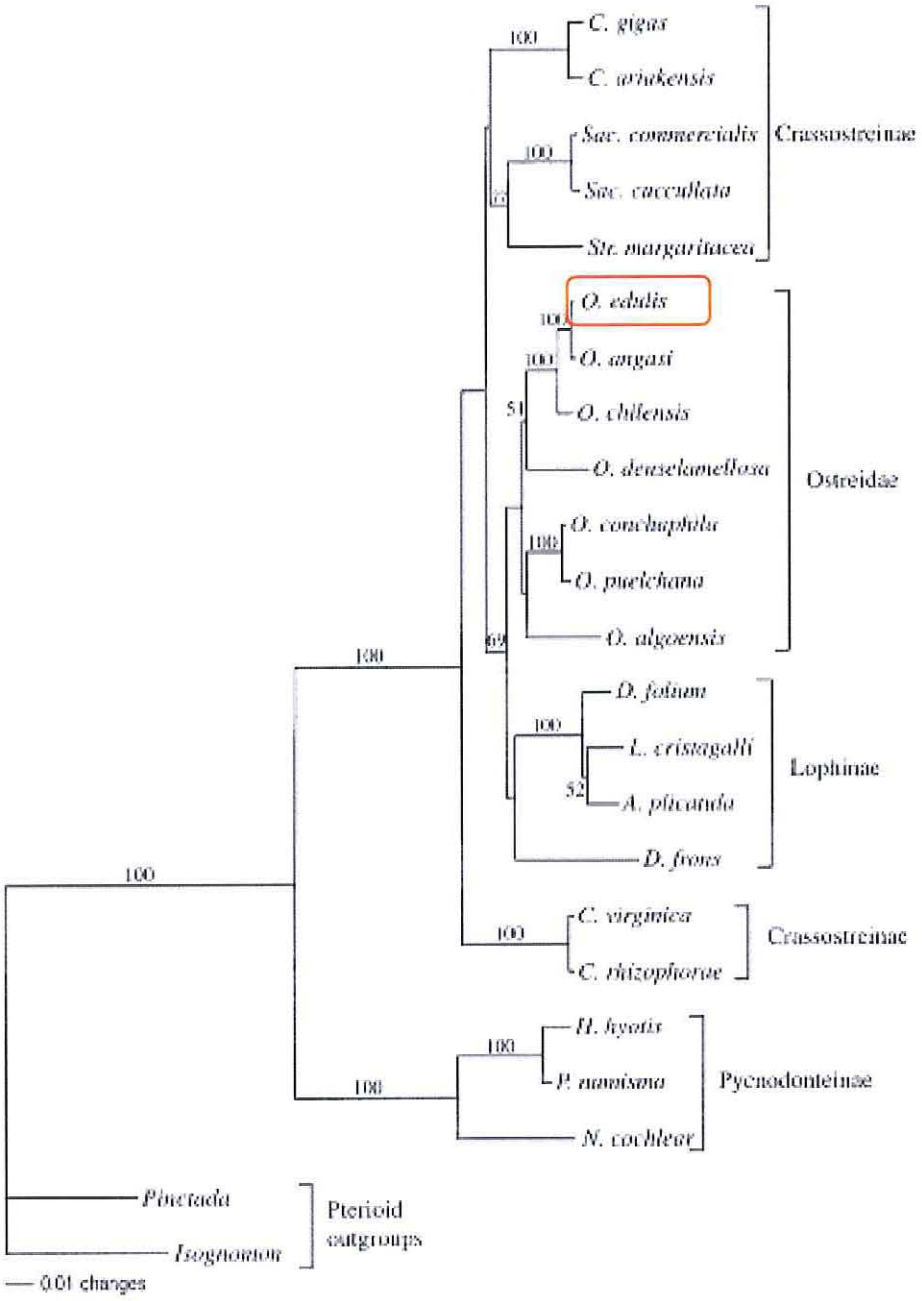
Appendix 1: Diagram world production.



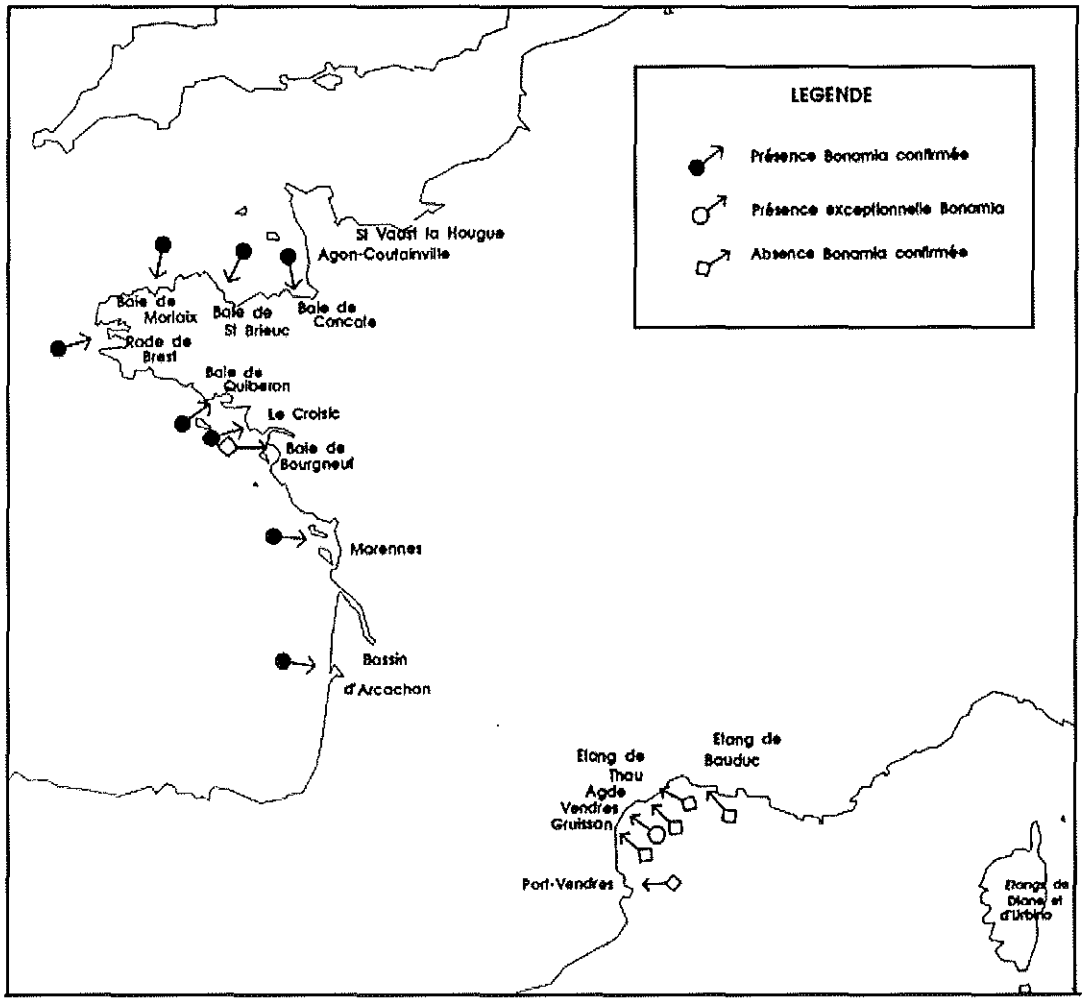
Appendix 2: Localization of the strategic points for the production of flats oysters in France.



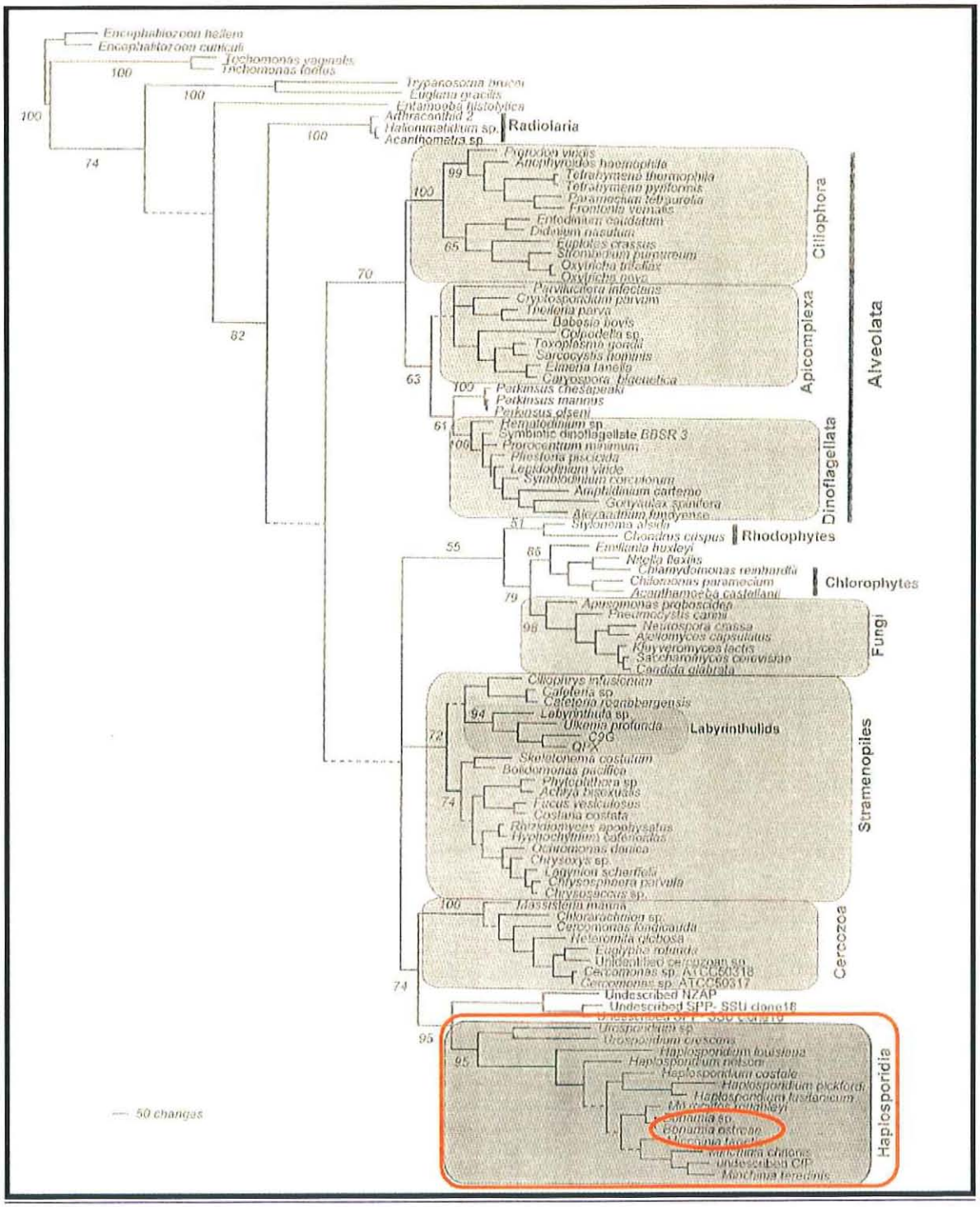
Appendix 3 : phylogeny of mollusc



Appendix 4: FAO, Chart of presence-absence of the parasite Ostrea edulis on the flat oysters of the French coasts (1990).



Appendix 5: Phylogenetic classification of *Bonamia ostreae*
(Reece *et al.* 2004)



Appendix 6: genes identify by B.Morga.

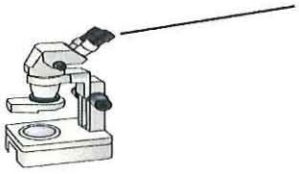
Brespiratory chain	Cytochrome Oxydase III P450
Cytoskeleton	Filamine MAPK ARP
Membran receptor	Receptor Lipo Galectin
Detoxification	Tetraspanin SOD OGST
Stress Protein	HSP
Immune system	TIMP
Protein of regulation	Clathrin SRC

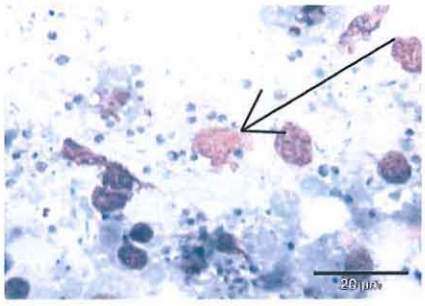
Annexe 7 : *Bonamia ostreae* parasites

Purification de *Bonamia ostreae*

-Réalisation de frottis cardiaque (Cf protocole) à partir d'une hutrître plate.

-Observation du frottis au microscope afin de déterminer le niveau d'infestation de l'huître par le parasite.





-Cellules huître plate infesté par Bonamia ostreae.

-Rincer l'intérieur de l'huitre avec EMFT.

-Mettre le corp de l'huitre dans le potter pour le broyer à l'ultra turax.

-Passer le bouillon sur un tamisde 250µm, puis sur un tamis de 75µm et 25µm.(Rincer chaque tamis)

-Placer le bouillon dans 5 tubes Falcon 5OmL environ 30-40mL de solution par tube.

-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Vider les tubes par retournement, remettre le culot en suspension dans 3ml d'EMFT.
-Rincer les tubes avec 2mL d'EMFT, regrouper tous les tubes dans un seul tube et passer ce dernier à l'ultra turax vitesse trés lente. Rincer la tige de l'ultra turax de manière à avoir un volume final de 40mL. Répartir ce volume en 4 tubes sur un coussin de 25mL de sucrose 20%.

-Déposer la solution à la pipette de manière à ne pas émulsioner les deux phases

-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Vider les tubes par retournement, remettre le culot en suspension dans 2ml d'EMFT.
-Rincer les tubes avec 1ml d'EMFT, regrouper tous les tubes dans un seul tube et passer ce dernier à l'ultra turax vitesse trés lente. Rincer la tige de l'ultra turax de manière à avoir un volume final de 20mL. Répartir ce volume en 4 tubes sur un gradient de sucrose 40%-20%. (25ml de 40% et 12ml de 20%)

-Réaliser les gradients de sucrose avec la pompe périsaltique.

-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Prélever l'interface 20-40 et rincer volume à volume dans un tube à fond rond avec de l'EMFT. (Mélanger doucement)

-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Préparation des gradients de percoll, à l'aide de la pompe périsaltique déposer 2,5mL de chaque solution par tubes. (tube conique Falcon 15mL)

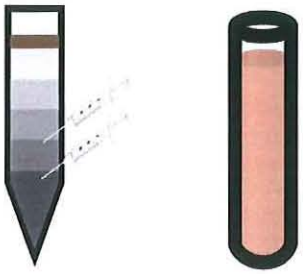
-Le nombre de tube de gradient dépend de la turbidité de la solution à déposer.

-Vider les tubes par retournement reprendre les culots dans 1 mL d'EMFT ,regrouper dans un seul tube rajouter le volume d'EMFT nécessaire de manière àdéposer 1mL sur les gradients de percoll.

-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Prélever les interfaces 60-70 et 50-60 à l'aide d'une seringue et rincer volume à volume avec de l'EMF (SANS TWEEN) 13mL volume maxi.

-Déposer sur un coussin de sucrose 20% (sans tween) environ 1mL (Tube conique Falcon 15mL).



-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

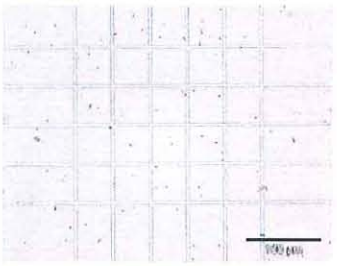
-Vider les tubes avec une pipette en laissant un peu de liquide au fond reprendre les culots dans 1mL regrouper dans un seul tube.

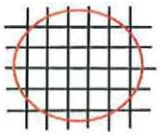
-Equilibrer et centrifuger à 8°c pendant 30min et 3500 tr

-Vider le tube avec une pipette, diluer le culot dans 500µL d'EMF et rincer le tube avec le même volume afin d'avoir un volume final de 1mL.

-Faire une dilution au dixième 10µL de solution de bonamia dans 90µL d'EMF.

-Conpter cette dilution sur cellule de Malassez.
(laisser quelques minutes sédimenter les cellules)





-Compter 3 rectangles de 20 petits carrés et faire la moyenne pour obtenir la concentration de la solution tenir compte la dillution.

-Solutions à préparer

Eau de mer fitré Tween (EMFT) :

-Eau de mer filtrée + 1% de Tween 80%.

Rmq : Prévoir 1,5L pour une manipulation

Sucrose 40% :

-40g dans 60mL EMFT

Sucrose 20% :

-20g dans 80mL EMFT*

*Attention car pour le dernier coussin il faut prévoir de l'eau de mer sans Tween à diluer avec le sucrose

Solution de Percoll :

Pour 9 tubes

-60mL de Percoll (frigo)

-2.46g de NaCl

Gradients de Percoll :

Pour 9 tubes

	Percoll (mL)	EMFT (mL)
30%	6.75	15.75
40%	9	13.5
50%	11.25	11.25
60%	13.5	9
70%	15.75	6.75

Appendix 8 : Plasmidic extraction

Extraction Plasmidique

- Centrifuger la culture bactériennes de 5 ml à 3 000g pendant 15 minutes à 24°C.

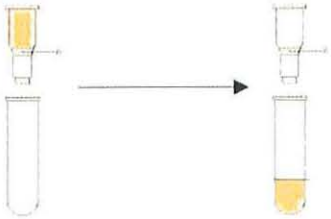
- Vider le surnageant par retournement.

- Ajouter 400ml d'ICE COLD complete lysis solution.

- Vortexer 30 secondes et incuber à 24°C pendant 3 minutes.

- Transférer la solution dans une spin colonne.

- Centrifuger 60 secondes à 10 000g



- Ajouter 400ml de wash DNA.

- Centrifuger 60 secondes à 10 000g..

- Transférer la colonne dans un nouveau tube.

- Ajouter 50 micro litres de Tampon d'élution au centre de la colonne.

- Centrifuger 60 secondes à 8 000g.

- Passer au spectromètre avec une dilution de 5microlitres dans 95 micro litres d'eau Sigma.

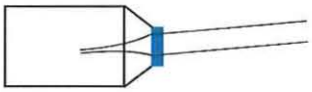
Appendix 9 : RNA Extraction

Extraction d'ARN

Jour 1

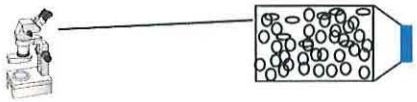
-Prélever l'hémolymphe (cf protocole)

-Déposer 5mL d'hémolymphe par fiole. (2 millions de cellules/mL)



-Incuber 2h30 à 15°C

-Observer au microcope pour voir si le tapis cellulaire s'est formé.



-Retier le surnageant doucement pour ne pas abîmer le tapis cellulaire, placer ce surnageant dans un tube Falcon 15mL. (Laisser environ 500µL dans la fiole)

-Déposer 500µL de la solution de Bonamia (50 millions de cellules)dans les fioles afin de réaliser la mise en contact, pour les fioles "temoin" déposer 500µL d'eau de mer filtrée.

-Laisser 2h à température ambiante (20°C).

-Pendant la mise en contact filtrée l'hémolymphe à 0.22µm.

-Réintroduire l'hémolymphe dans les fioles et laisser toute la nuit à 15°C.

Jour 2

-Retirer le surnageant des fioles.

-Laver la fiole avec du PBS 1X froid 4°C environ 5mL répéter l'opération.

-Ajouter 1mL de Trizol, agiter doucement la fiole et récupérer le lysa dans un tube 1.5mL.

-Laisser le lysa à température ambiante ou bien le congelé pour conservation (-20°C).

-Ajouter 1/5ème de chloroforme isoamylique

-Centrifuger à 4°C pendant 10min et 12000 g

-Récupérer la phase acqueuse.

-Ajouter un volume d'Isopropanol froid (4°C).

-Incuber 30 minutes à 4°C.

-Centrifuger à 4°C pendant 30min et 12000 g

-Eliminer le surnageant et laver le culot avec 1mL de l'éthanol 70%.

-Centrifuger à 4°C pendant 10min et 12000 g

-Eliminer doucement le surnageant.

-Centrifuger à 4°C pendant 2min et 12000 g

-Eliminer les dernières gouttes d'éthanol.

-Laisser les tubes sécher sous la sorbone.

-Reprendre les culots dans 22µL d'eau DEPC.

Control sur gel

-Utiliser la cuve spéciale ARN.
-Rincer tout le matériel avec de l'eau oxygéné à 3 volumes (1 heure).
-Rincer avec de l'eau DEPC.

-Préparer du tampon TAE 1X avec de l'eau DEPC.

-Préparer un petit gel à 1% d'agarose freeARN (0.5g d'agarose dans 50mL de TAE et 2,5µL de BET).

-Marqueur de taille ARN.

-Déposer 5µL de produit d'extraction et 2µL de bleu de charge.

-Migration 50 Volt.

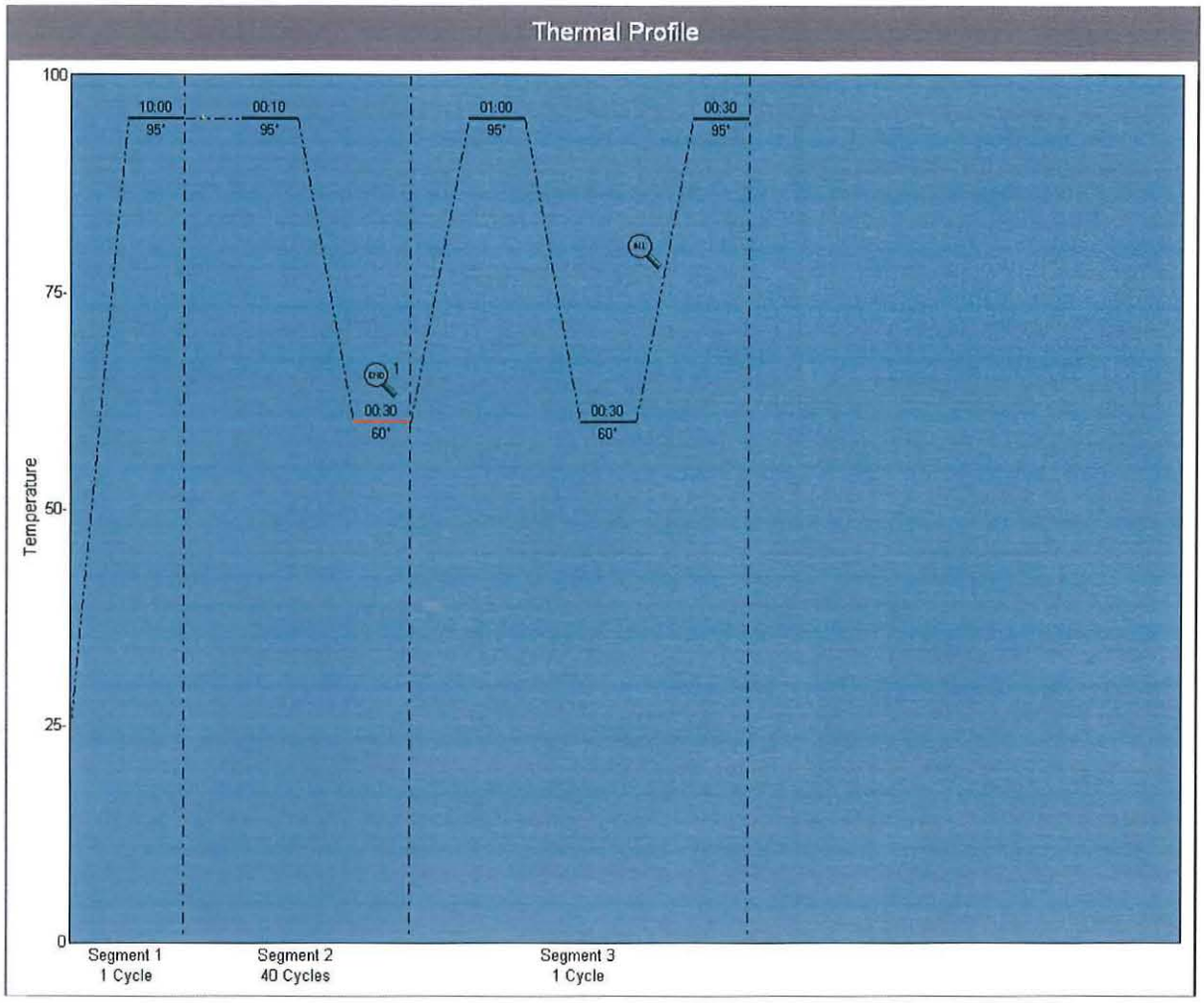
-Control du gel à la plaque ultra violet.

Control au spectro

-Réaliser un blanc.

-Dilution 2µL de produit d'extraction + 98µL d'eau DEPC.

Appendix 10 : Profile thermique of QPCR



Appendix 11: Principe of SYBR GREEN for QPCR