CONTRACT No AQ1-272

STUDIES OF A EUROPEAN SHELLFISH DISEASE : *BONAMIA OSTREAE* PARASITE OF THE FLAT OYSTER *OSTREA EDULIS* ; CONTROL AND TREATMENT

FINAL REPORT

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("FAR")

GENERAL INTRODUCTION

Within the oyster *Ostrea edulis* in France (Ile Tudy, South Brittany), 1979, a protozoan parasite was described as *Bonamia ostreae* (1).

On the basis of ultrastructural characteristics, principally the presence of haplosporosomes, this parasite has been classified with the phylum Ascetospora. The development cycle of *Bonamia ostreae* has been studied by electron microscopy (2), and it appears relatively simple. Unicellular forms of the parasite multiply by scissiparity in host haemocytes which, after disrupting, release the parasites. Plasmodial forms can be observed most frequently in dying animals. There is no sporulation in the oysters and the existence of spores remain uncertain. Indeed, the available evidence suggests a life-cycle with no intermediate host (3).
The transmission of the disease occurs throughout the year. Since 1979, bonamiasis has spread into all the breeding areas of Brittany, leading to a 90% drop of the productions. As a result of numerous national and international transfers the disease then appeared progressively in several other European countries; Spain, England, Holland, Ireland and in the USA. The disease has led to economically disastrous losses of production in these countries.

The scale of the promblem has made it essential to establish zoosanitary controls. The spread of the disease has been regularly monitored and the precise epidemiological status of oyster-growing areas in the different countries has been established (PART I).

In order to be able to effectively limit the impact of the disease research in the field was also required to resolve the influence both of some zootechnical and environmental parameters on the bonamiasis and the development cycle of the parasites (PART II). The establishment of prophylactic measures strongly depends on knowledge, at molecular and cellular levels, of the effects of environmental parameters in the oysters' physiology, biochemistry and immune systems which increase their susceptibility to *Bonamia ostreae* (PART III). The relationship of the parasite with its host, in the field at individual level, but also at cellular level, must be consequently also explained (PART IV). These relations require study of the defence mechanisms of the oysters and their interactions with parasites (PART V). The understanding of immune capacities and the establishment of some clue of defence capacity, might lead to determine criteria of resistance. Indeed, beside passing zootechnical measures and prophylaxy, the pereniality of the productions is strongly linked to the selection of resistant strains of oysters which constitute an important way of research to develop (PART VI).

As proposed in the contract, the different main purposes of the research were:

- to establish an accurate epidemiological picture of the bonamiasis in different *Ostrea edulis* european breeding areas.(PART I- J. Mc Ardle, J. Mazurie and J. Montes),

- to determine the influence of zootechnical and environmental parameters on the development of the disease in the field (**PART II - N. Cochennec, S. Culloty, J. Mazurie, J. Montes and M. Mulcahy**) and at molecular and cellular level (**PART III - L.E. Hawkins and S. Hutchinson**).

- to study humoral and cellular defence reactions (**PART IV - S. Culloty, A. Figueras and M. Mulcahy**) and understand the host-pathogen interactions at cellular level and the mechanisms of recognition, elimination and escape of the parasite (**PART V - E. Bachère, V. Boulo, D. Hervio and E. Mialhe**).

- to assess relative resistant oyster strains in the aim of selection (**PART VI - E. Bachere, V. Boulo, N. Cochennec, S. Culloty, D. Hervio, E. Mialhe and M. Mulcahy.**

PART I

DESCRIPTIVE EPIDEMIOLOGY

J.Mc ARDLE, J. MAZURIE and J. MONTES

CONTENTS

CHAPTER 1

INTRODUCTION

Following the initial outbreak in Brittany in 1979, the disease subsequently spread to the Netherlands (1980), England (1982) and Spain and Ireland (1987) invariably causing significant losses and the severe curtailment of flat oyster production in the affected areas. During 1990-91, the epidemiological survey of the bonamiasis around the European coast has been particularly carried out in Galicia (Spain), in Brittany, north of France and in the west coast of Ireland.

In Spain, the estuaries of Ribadeo, San Ciprian, Ortigueira, Sada, Noya, and Arosa were monitored for the presence of *Bonamia*. The geographical location of the different sites is shown in figure 1.

The parasite is known to be present in the main areas in France apart from the Baie de Bourgneuf and the Mediterranean. The zoosanitary state is shown in figure 2 in terms of presence-absence. It may be noted that a detailed epidemiological survey has been carried out in Brittany as part of a regional stimulation project. Since the outbreak of the parasites *Marteilia refringens* and *Bonamia ostreae* the breeding activity of flat oysters is essentially practised in the three deep areas of Baie de Cancale, St Brieuc and Quiberon (fig. 3).

The epidemiology of the bonamiasis in Ireland can be divided into three major phases (fig. 4).

Outbreak 1 - Cork Harbour. The disease was first diagnosed in oysters being intensively cultivated in Cork Harbour in early 1987. Significant mortalities occurred in virtually all year classes except the 1 year olds.

Outbreak 2 - Clew Bay. In January 1988 oysters dispatched from Clew Bay, a large deep water site, for relaying to France were found to be positive for *B. ostreae* when examined on arrival in France. However, despite the presumptive identification of the parasite in oysters, there were no obvious mortalities and in the intervening period this situation has remained unchanged.

Outbreak 3 - Galway Bay. *B. ostreae* occurred in Galway Bay in 1989. Mortalities began in a small inlet in inner Galway Bay where oysters dredged in the bay had been relaid. Mortalities appeared to commence around the end of August and examination of impression smears indicated a prevalence of about 50%. Mortalities of 70-80% were reported in this outbreak. The source of the infection may have been oysters from Cork as a consignment of oysters was transferred from Cork to Galway in 1985 and 1986.

Following the outbreak of bonamiasis, the spread of the disease has been regularly monitored in the three countries.

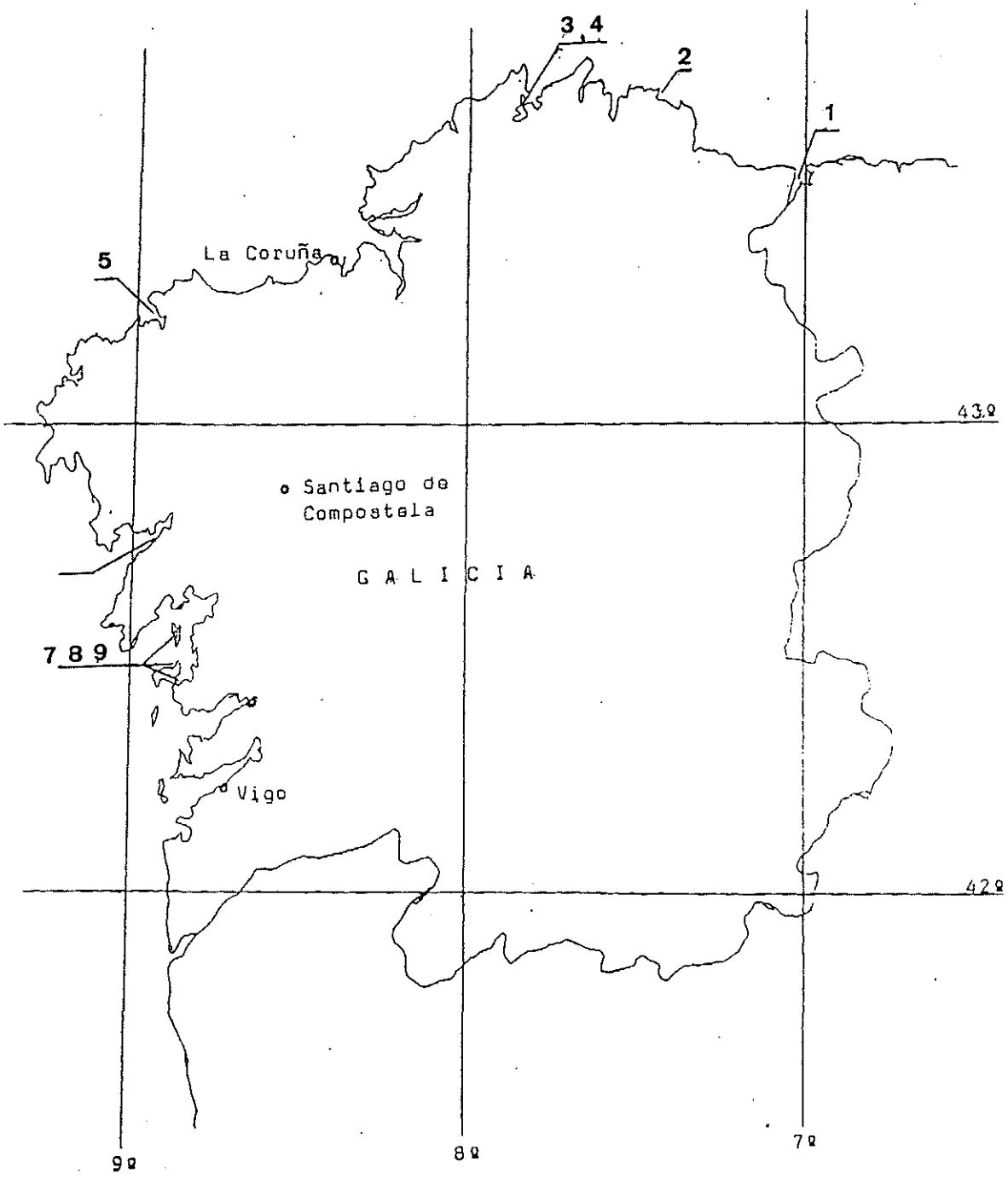


Figure 1. Geographical location of the sites sampled on Galician Coast (Spain).

1: Ribadeo; 2: San Ciprian; 3-4: Ortigueira; 5: Sada; 6: Noya; 7-8-9: Arosa.

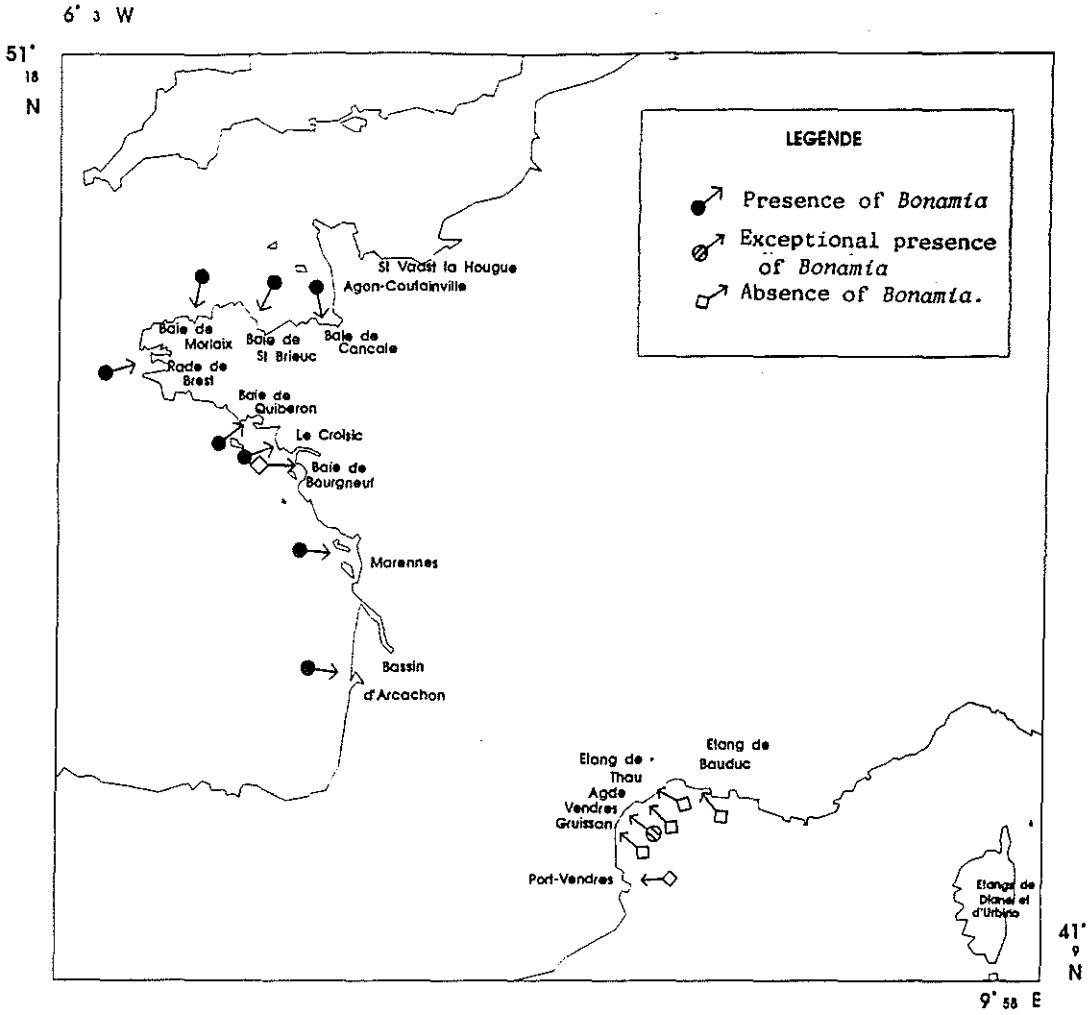


Figure 2. Zoosanitary state of main areas in France

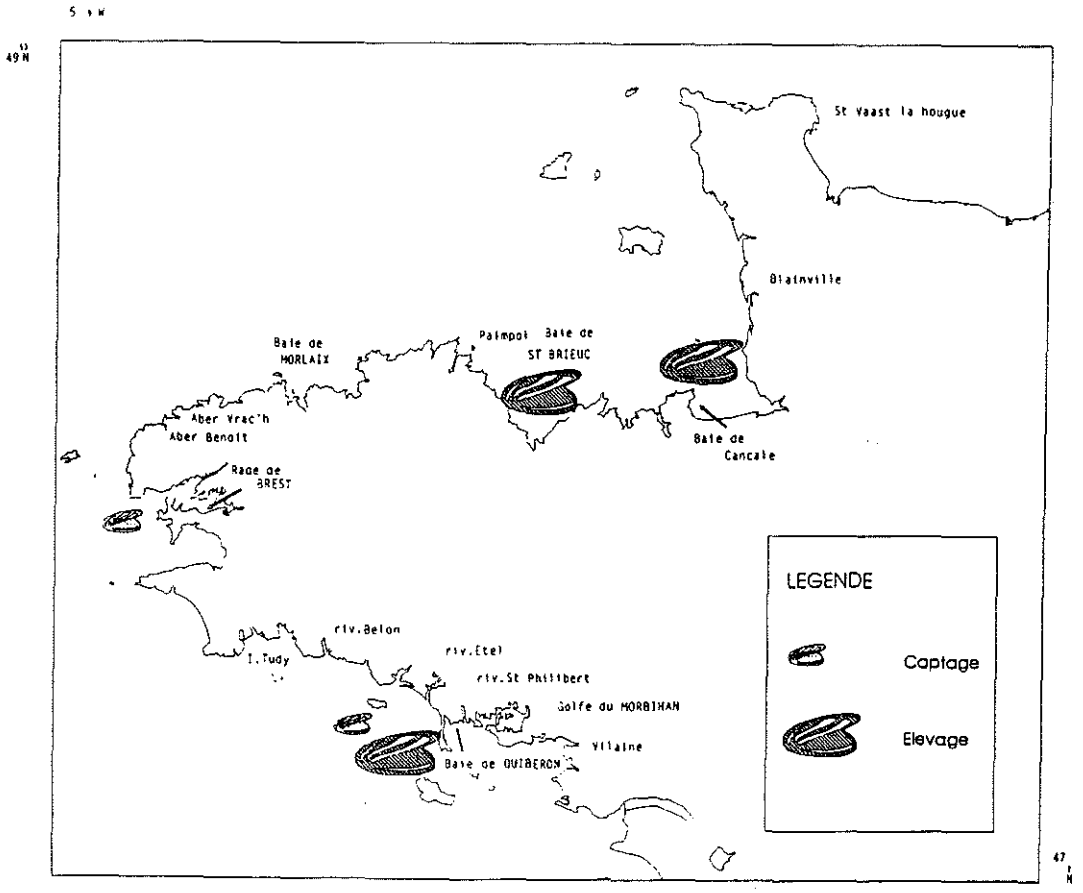


Figure 3. Main sites for settlement and rearing of flat oysters in Brittany (France).

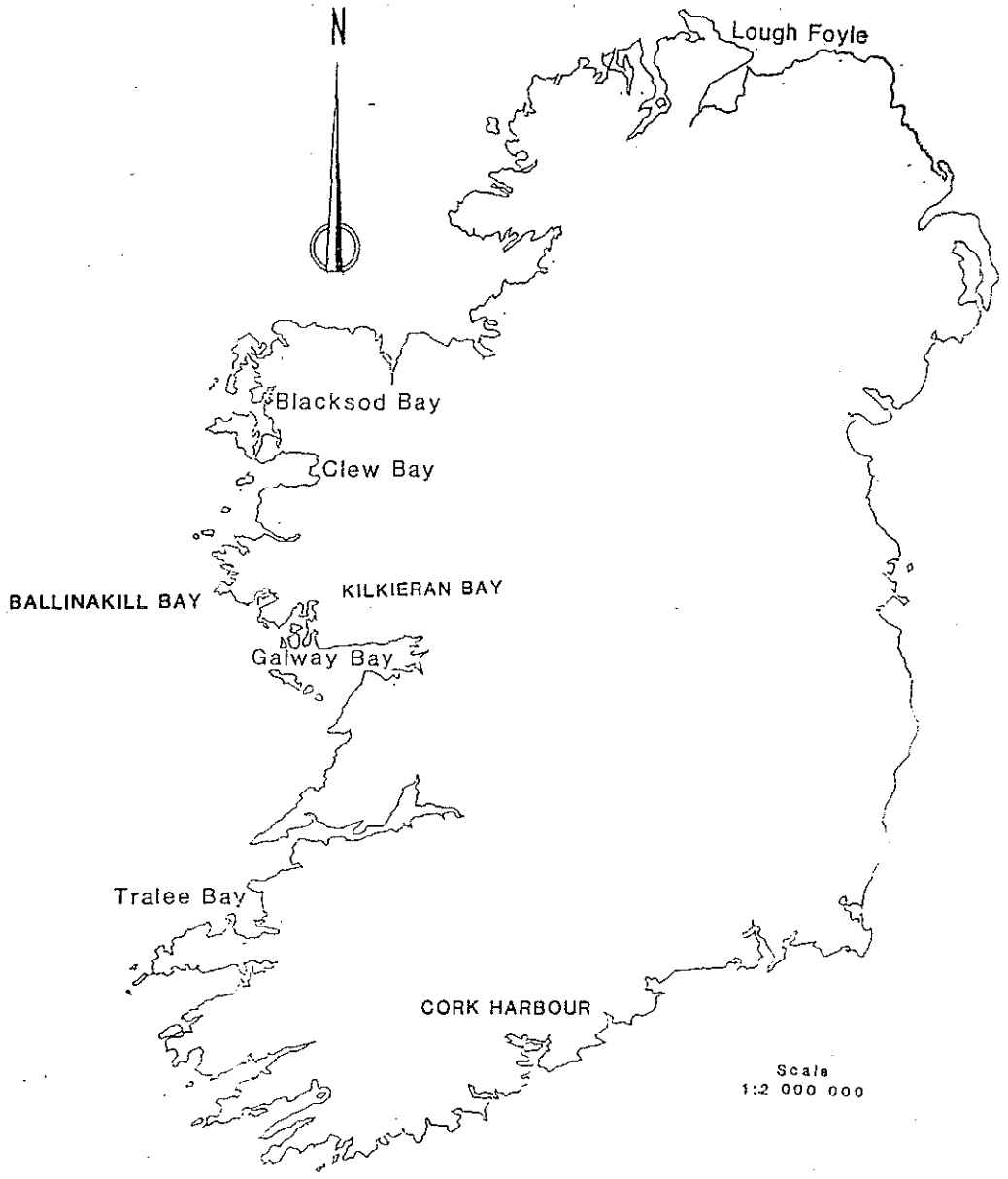


Figure 4. Map showing principal oyster growing areas in Ireland.

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLING LOCATIONS

In Galicia (Spain), the areas of Arosa (3 sites: Island, O Grove and Noalla) and Ortigueira (Fornelos and Macelos), where the oyster culture is well developed, were sampled every 3-4 months, and once or twice a year in the other areas (fig.1).

Two controls, in spring and in autumn, are annually performed in the French breeding sites of St Michel Bay (Cancale), St Brieuc Bay and Quiberon Bay. The age, weight and the number of the sampled oysters are presented in the table 1 for the different three bays.

Most of the important flat oyster growing areas in Ireland surveyed for the presence of the disease are Cork harbour, Tralee Bay, Galway bay, Clew Bay, Kilkieran bay, Ballinakill bay and Lough Foyle (Fig 4).

The layout of the oyster beds in Cork harbour is illustrated in figure 5. In Clew Bay (fig 6), 8 to 12 stations were surveyed annually. For Galway Bay 6, stations surrounding the immediate area of the original outbreak were surveyed at distances of approximately 100 metres from the preceding station (fig 7). In 1990, 13 sites were sampled with a total of 2,228 oysters being examined. In 1991, 12 sites in Galway Bay were sampled with usually between 100 and 150 oysters per sample being examined. Tralee Bay was sampled twice yearly at least. Finally, two samples from Kilkieran Bay were examined in 1990 and 1991.

The oysters beds, which total 26, were dredged. Both the number of dead oysters ("clocks") and live oysters were determined to establish the overall prevalence of the disease. The level of mortality and infection was related to the age of the oysters. Histological examination of 150 live oysters from each bed was carried out to estimate the level of infection in the surviving oysters.

2.2 METHODS FOR DETECTION OF B.OSTREAE

2.2.1 Impression smears

Oysters were opened and the heart was removed and placed on dry filter paper to eliminate excess haemolymph. The dark atrial portion of the heart was separated from the paler ventricle and the ventricle was pressed on to a clean dry slide to make a series of impressions on the slide. The slides were then air-dried, fixed with methanol and stained using a "Hemacolor kit" (Merck) by immersion for 30 seconds in each reagent. The slides were examined by optical microscopy for the presence or absence of the parasite. From 1989 onwards this was the method commonly used for detecting the disease.

2.2.2 Histological examination

A slice of oyster tissue was taken from each oyster through the visceral mass including an attached piece of gill and mantle. The tissue

Sampling date	Spring 1990		Autumn 1990		Spring 1991		Autumn 1991	
	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate
1987	/	2/51						
1988	/	/	/	/	/	0/51	/	3/50
1988	/	1/100	50 g	5/100	42,2 g	0/50		
1989	before /	seeding 1/261	18 g	3/100	20,4 g	2/50	47,3 g	3/50
1989	before /	seeding 0/253	/	/	23,0 g	0/50	45,0 g	0/50
Young oysters	/	/	/	/	14,3 g	1/73	/	/
commerc. oysters	/	/	/	/	95,6 g	3/94	/	/
1990					before /	seeding 0/150	11,8 g	0/50
1990					before /	seeding 0/100	15,0 g	0/50

1. CANCALE BAY.

Sampling date	Spring 1990		Autumn 1990		Spring 1991		Autumn 1991	
	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate
1988	/	/	59 g	45/100	53 g	54/76		
1989	before	seeding 0/89	11 g	0/50	/	/	/	/
1989	before	seeding 0/50	10 g	0/50	20 g	0,50	/	2/50
1989	before	seeding 0/150	13 g	0/100	/	2/95	56,5 g	4/100
1990					before /	seeding 0/95	/	0/50
1990					/	/	10,7 g	0/50
1990					before /	seeding 0/150	/	0/50
1990					before /	seeding 0/100	/	1/50

2. ST-BRIEUC BAY.

Sampling date	Spring 1990		Autumn 1990		Spring 1991		Autumn 1991	
	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate	Average weight	Infection rate
1987	57,5 g	9/50	/	14/108				
1988	28 g	5/50	30 g	6/100	/	32/100	75,9 g	20/100
1989	before /	seeding 0/50	14 g	0/100	/	4/100	40,9 g	24/100
1990					before /	seeding 0/100	15,8 g	1/100
1991							before /	seeding 0/50
All age	/	32/100	/	5%	/	22/100	/	13/100

3. QUIBERON BAY.

Table 1. Evolution of infection rates of flat oysters in three areas of Brittany (France).

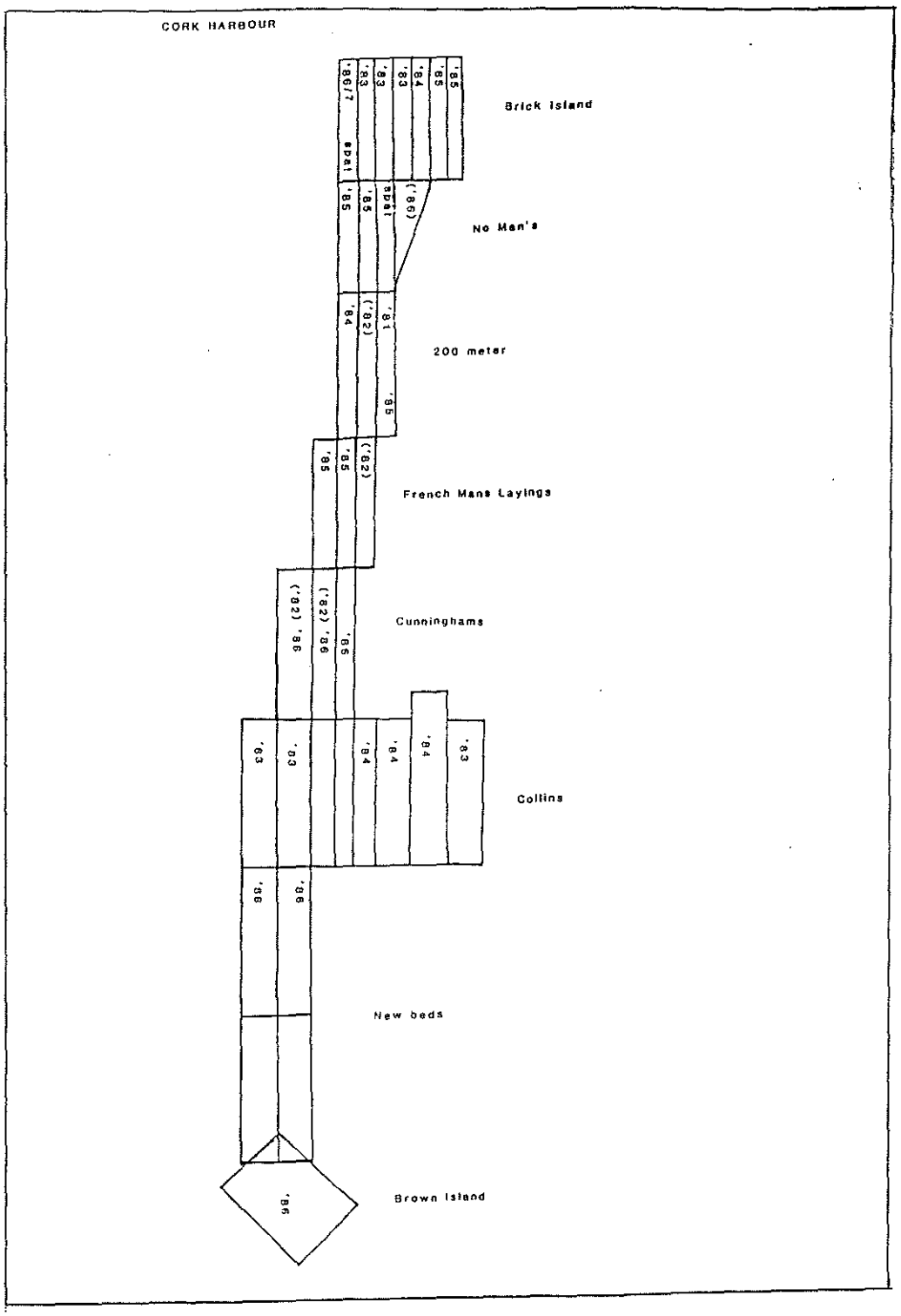


Figure 5. Diagram showing the layout of the oyster beds in Cork Harbour (Ireland).

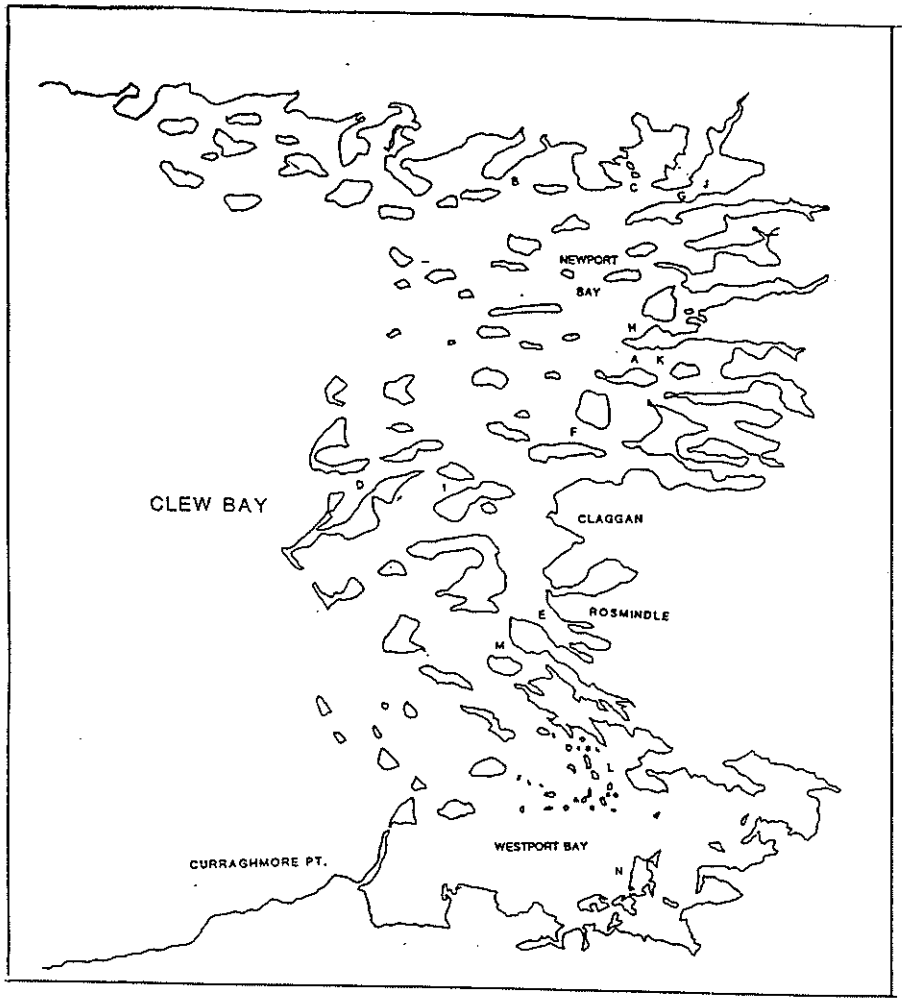


Figure 6. Bonamiasis epidemiological survey Clew bay 1990 (Ireland).

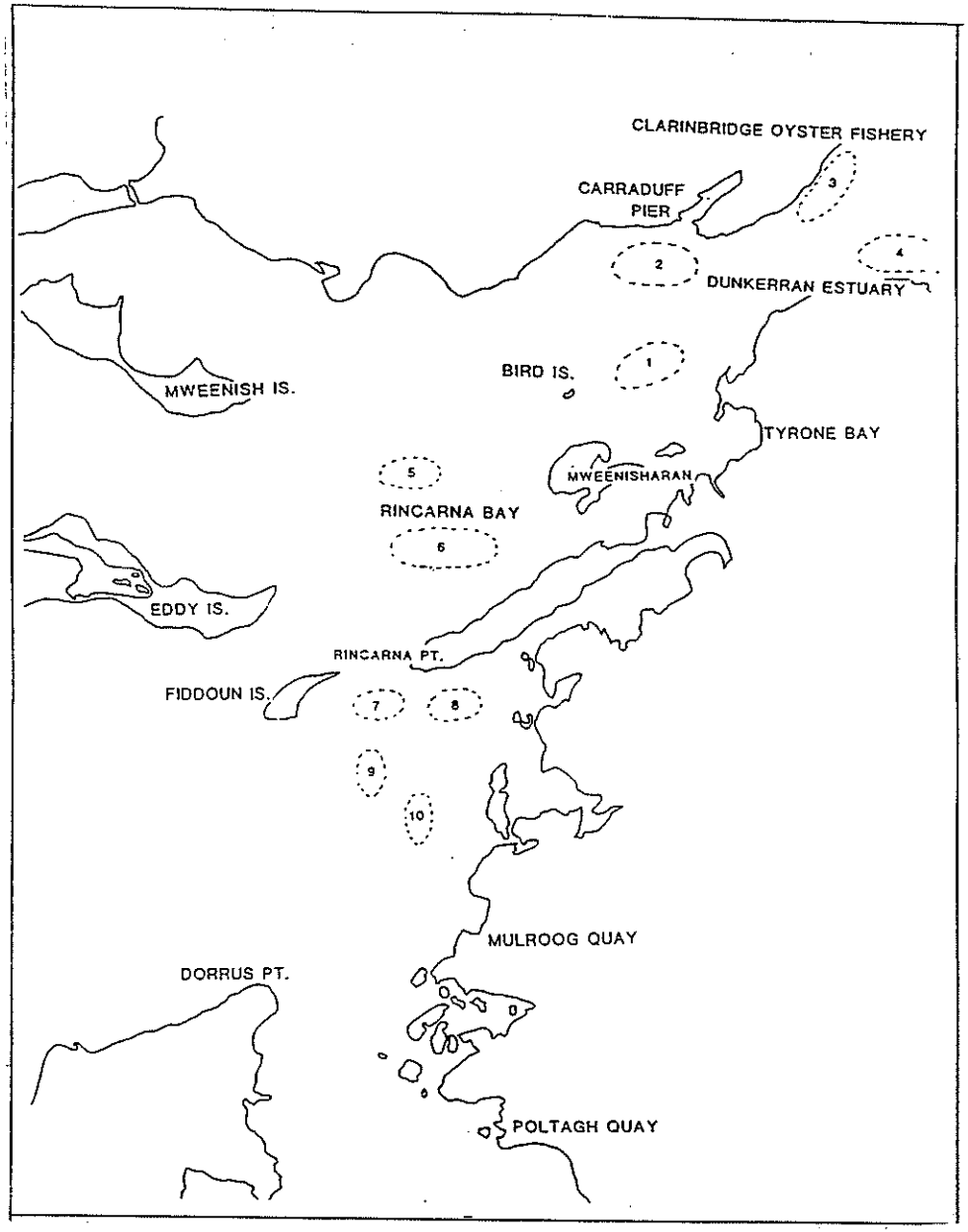


Figure 7. Map showing principal sampling stations in Galway bay (Ireland).

was fixed in modified Davidsons fixative for 24 hours and then transferred to 70% alcool and processed in the normal way for histological examination. 5 µ sections were stained with haematoxylin and eosin and examined.

2.2.3 ELISA test

The test is a commercial one (Sanofi), based on an enzyme linked immunosorbent assay method using the "sandwich" technique (4). After gently removing the excess of seawater by blotting, haemolymph was collected from the oysters with an Eppendorf micro pipette carefully introduced into the pericardium cavity. The haemolymphs were freeze at -20°C followed by thawing in order to disrupt the haemocytes. The samples being tested and the control reagents were distributed in the wells of a plastic microplate which was pre-coated with a monoclonal antibody prepared against the parasite. A monoclonal antibody linked to the enzyme alkaline phosphate was added after incubation. Following washing, the enzyme substrate was added and at the end of the reaction time the absorbance was read at 405nm.

2.3 A GEOSTATISTICAL METHOD is presented in chapter 3

CHAPTER 3

RESULTS AND DISCUSSION

3.1 EPIDEMIOLOGICAL STATE OF *B. OSTREAE*

SPAIN

Bonamia ostreae was found in Rebadeo (site 1), Ortigueira (sites 3 and 4), Sada (site 5) and Arosa (sites 7, 8 and 9). The infection rate was higher in the oysters reared for a long time (more than 6 months) than in those inmersed a short time. The table 2 shows the maximum infection rate found in each site.

Although *Bonamia ostreae* was not detected in San Ciprian (site 2) and Noya (site 6), only the estuary of San Ciprian can be really considered free of *Bonamia*, since the parasite has never been detected there. Nevertheless, in the estuary of the Noya the non-detection of parasite could be attributed to a decrease of the prevalence *B. ostreae* following a break in oyster culture in this estuary.

The high prevalences of *B. ostreae* found in the estuaries of Galicia, mainly in the areas where the culture of the flat oyster is intensive, cause high mortalities. The populations of flat oyster are usually decimated before they reach the marketable size. Under these circumstances, the culture of the flat oyster is umprofitable.
So, the areas where *Bonamia* is not present need to be protected against the introduction of "foreign" oysters and possible contamination.

ESTUARY	SITE OF SAMPLING	HIGHEST INFECTION RATE	PRESENT STATUS
Ribadeo	(1)	43%	infected
San Ciprian	(2)	0%	free of *Bonamia*
Ortigueira	Fornelos(3)	47%	infected
	Macelos (4)	73%	infected
Sada	(5)	3%	infected
Noya	(6)	0%	?
Arosa	Island (7)	67%	infected
	O Grove(8)	35%	infected
	Noalla (9)	17%	infected

Table 2.- Present status of bonamiasis in Galicia (NW of Spain).

IRELAND

Galway Bay

Using the heart impression smear test the disease was not detected at any new sites in Galway bay in 1990. Surveys carried out at increasing distances from the original infected site yielded negative results apart from one area very close to the original site of infection. However, using the ELISA test nine other sites that were negative on the smear test yielded a positive result.

In 1991 using the smear test the disease was detected at 5 new sites in Galway bay. The first occurred in May in Aughinish bay which is at a significant distance from the first disease outbreak in 1989. The disease was detected in wild oyster stocks at a low prevalence rate of approximately 2%. No significant mortalities were associated with this detection of the parasite and the oysters appeared normal. The oysters were between 4 and 5 years old.

In June 1991 the disease was detected at a second site in Galway Bay. The parasite was detected in a group of oysters which had been dredged in the Clarinbridge area and then stored in holding tanks (fig 7). The prevalence of the disease was extremely low, less than 1% and no significant mortalities were noticed. Finally, the disease was detected at three sites sampled in July and August (stations 8,9,10 fig 7). The prevalence in these cases was 2-3% and again no mortalities were associated with the finding of the parasite.

Cork Harbour

The disease in Cork harbour appears to be still relatively severe with a relatively high prevalence rate being recorded. In 1990 the prevalence was estimated at 34.8% in April and in 1991 at 45.6% in January.

Clew Bay

Following the detection of the disease in oysters from Clew Bay exported to France in 1988 a large number (>1400) oyster haemolymph smears were examined and failed to show evidence for the disease although one doubtful slide was retrospectively considered positive by IFREMER workers. The ELISA test detected a small number of positives (18 in all). The sampling stations are indicated in figure 6. In 1990 eight stations surveyed with haemolymph smears from a total of 959 oysters being examined, yielded no evidence of the disease. Although 20 oysters yielded positive results on the ELISA test.

Tralee Bay, Kilkieran Bay, Ballinakill Bay and Lough Foyle have been found *Bonamia*-free.

The pattern of bonamiasis outbreaks in Ireland show some similarities to those which have occurred in other countries but significant differences were also observed. The first outbreak of the disease in Cork harbour followed a similar pattern to bonamiasis outbreaks in France, the Netherlands and the UK with large scale mortalities being observed in market-sized, mature oysters followed by a significant curtailment of oyster production in the affected area. The source of the infection in Ireland is still unclear. At the time the disease first broke out in France the importation of flat oysters into Ireland from France was prohibited. The general belief is that the disease was introduced in an illegal consignment of oysters originating in France which came into the south-west of Ireland in the early 1980s. However, it is also possible that the parasite could have been present in Ireland for a good deal longer than

this and that the intensification of flat oyster culture in Cork harbour resulted in the disease being expressed in a much more serious way than would be the case in scattered wild oyster populations.

The first Galway bay outbreak resembled the outbreak in Cork harbour in that the mortality rate was high with the highest losses occurring at the end of the summer. It is also noteworthy that the serious losses experience occurred in oysters being held at high stocking densities. However, the first disease outbreak in Galway was much more focal than the Cork outbreak with detection of the disease outside the immediate affected are a being virtually impossible. This was probably due to the fact that, although the site in which the disease occurred was an area of high density oyster relaying, the bulk of flat oyster production in Galway Bay is obtained by harvesting the extensive wild oyster beds there, unlike in Cork where oysters were intensively reared over a large area.

Following the initial outbreak in Galway bay the subsequent surveys carried out there yielded interesting results regarding the pattern of spread of the disease and its severity in wild oyster populations. The fact that it was virtually impossible to detect the parasite outside the immediate area of the first disease outbreak and that the second outbreak occurred some distance from the first one tends to suggest that the spread of the disease is more complex than by simple extension from an affected area. It is possible that factors such as water movements and currents may have a significant part to play in distributing the parasite from infected areas to more distant sites.

However, the question of spread of the disease in Galway Bay and other areas is further linked to man's activity, particularly that of transferring live oysters both between areas and within areas. The records show that a transfer of oysters from Cork harbour to Galway Bay took place in 1985 and 1986. These transfers probably inadvertantly contained some oysters infected with the disease, thus providing new foci of infection in Galway Bay. The custom of fishing for oysters and transferring stocks to buyers' storage areas in different parts of the bay probably also serves to disseminate the disease more widely. The situation is probably further exacerbated by the relatively short fishing season permitted which leads to buyer's purchasing relatively large quantities of oysters and immersing them in large numbers in confined areas thus providing ideal conditions for disease outbreaks and further spread of the disease.

It is also interesting that the survey of the wild oyster stocks in Galway Bay, although pin-pointing new areas of infection, also indicated a much lower disease prevalence with few if any associated mortalities being recorded. It is probable that in these new sites the disease would have largely gone unnoticed except for the large-scale sampling programme. Apart from the initial high mortality in a small confined area of Galway Bay, significant subsequent mortalities in wild oysters in the bay have not occurred and, overall, oyster production in Galway does not appear to have been significantly affected. This is one of the hopeful findings arising from the study. However, it may be that over a longer time scale flat oyster production in the area may decline as the disease tightens its grip on the oyster populations there. Whatever the ultimate outcome the studies shows that the expression of the disease in wild oysters populations appears to be significantly different from that observed in intensively cultivated stocks.

The disease situation in an other area, Clew Bay, appears to be even more complex since initial detection of the disease. The examination of a prodigious number of haemolymph smears has shown virtually no evidence of the disease although the ELISA test did detect a small number of positives (a total of 18). Classical histological methods have never yielded evidence of the disease there and no mortalities have ever been reported from there. The results obtained raise questions regarding the preferred test for use in detecting the disease. In dealing with clinical disease outbreaks classical histological methods are adequate to detect the disease and have the advantage that other diseases apart from bonamiasis are also detectable. Examination of haemolymph smears appears to be more sensitive in detecting the disease when the prevalence of the disease is low and clinical signs are absent. However some workers have pointed to the possibility of confusion of the parasite with other intracytoplasmic inclusions (6). This possibility cannot be ruled out in relation to the Clew Bay results although the results could also indicate an extremely low level of infection in an exclusively wild population of oysters.

The ELISA test in our hands tended to further confuse the issue rather than clarify it. The number of positive cases the test yielded was always consistently higher than any of the other two tests and led to problems of interpretation since many of these positive results could not be verified by any of the other methods. In the absence of any clinical evidence of disease or mortalities together with negative histological and smear tests, the recording of relatively high numbers of ELISA positive results particularly to Galway tended to suggest that some false positive results were being obtained. However it is worth pointing out that the ELISA test detected one oyster positive for the disease at the site of the second disease occurrence in Galway bay almost two years before it was detected there using the other two methods showing that the test may be much more sensitive than the other methods.

Apart from stocking densities there would appear to be a definite seasonality in relation to the occurrence of the disease. This is probably related to water temperatures as is the case in other parasitic disease of fish. The majority of the disease outbreaks and disease detection occurred in the late summer or autumn. This probably results from a gradual build of infection during the summer months as the water temperatures rise. In 1991 the summer was average and water temperatures did not reach very high levels. In Galway bay the water temperatures averaged about 16°C with highest levels of 18°C being recorded. This may have been important in explaining the lack of significant mortalities in the three disease outbreaks which occurred there. This seasonality in the occurrence of the disease could also be related to the sexual development of the oysters, (7) has shown a possible relationship between the occurrence of the disease and the sexual development of the oysters. However, further studies are needed to clarify this relationship (see PART II).

FRANCE

During 1990 and 1991, the epidemiological state of *B. ostreae* has differently developed in the three bays as shown in table 1.

In St Michel Bay which is the main breeding area, the infection rates observed during 1990-91 were less than 6% for 3 years old oysters originally sowed as seeds. Only, two oyster batches which have been handled (raising, grading, sorting and again sowing), have shown 13% and 14% of infection (table 1).

In St Brieuc Bay, during the same period, one batch, seeded in 1988, have revealed high infection rates, reaching 45% at the age of 2 and 71% few months later. The oyster batches subsequently scattered showed only 4% infection rate at the age of 2. A batch showed a prevalence of 20% consecutively to a transfer (table 1).

The bonamiasis developed in Quiberon bay more quickly during 1990 and 1991 when compared to the previous years. The higher infection rate reached 32% for 2 year-old oysters. This increase could be explained by the presence in 1989 of older oysters or stemming from transfered oysters with high prevalence.

To sum up, in the main breeding areas of Brittany, the zoosanitary state remains stable. The stocks increase possibly leading to an increase of the production in Brittany. In the two other deep water sites, the evolution of the production present some pronounced variabilities making more uncertain the breedings.

3.2 GEOSTATISTICAL METHOD FOR STUDY OF SPATIAL REPARTITION OF *BONAMIA OSTREAE*.

A geostatistical method was applied to the cultivated bed of *Ostrea edulis*, situated in the Bay of Quiberon (South Brittany, France). These oysters, seeded in 1986, showed a progressive infestation by *Bonamia ostreae*, reaching a mean prevalence of 23 % in 1989.

The distribution of thetotal number of oysters, and parasited oysters was studied by a geostatistical method, using BLUEPACK software (Geovariances Company). At this time, systematic samplings were performed inside the 60 m x 40 m oyster bed, at 2 scales : 10 m all over the area, and 1 m within a 100 m2 square (fig. 8). The modelling of variograms of the number of parasited oysters led to definition of spatial structures (fig. 9) and maps showing isolines of densities and *Bonamia* prevalence, at small scale (fig.10).

The total and parasited numbers of oysters show lengthened structures east-west oriented, probably reflecting the seeding practices. However, the distribution of parasited proportion appears uniform, whatever the local density of oysters.

The absence of alink between oysters density and instantaneous *Bonamia* prevalence could be the result of differential mortalities during the 3 years of rearing.

However, these geostatistical methods which provide sufficiently intense sampling to be used in the future for detailed studies of the distribution of oysters and parasites repartition in a growing area.

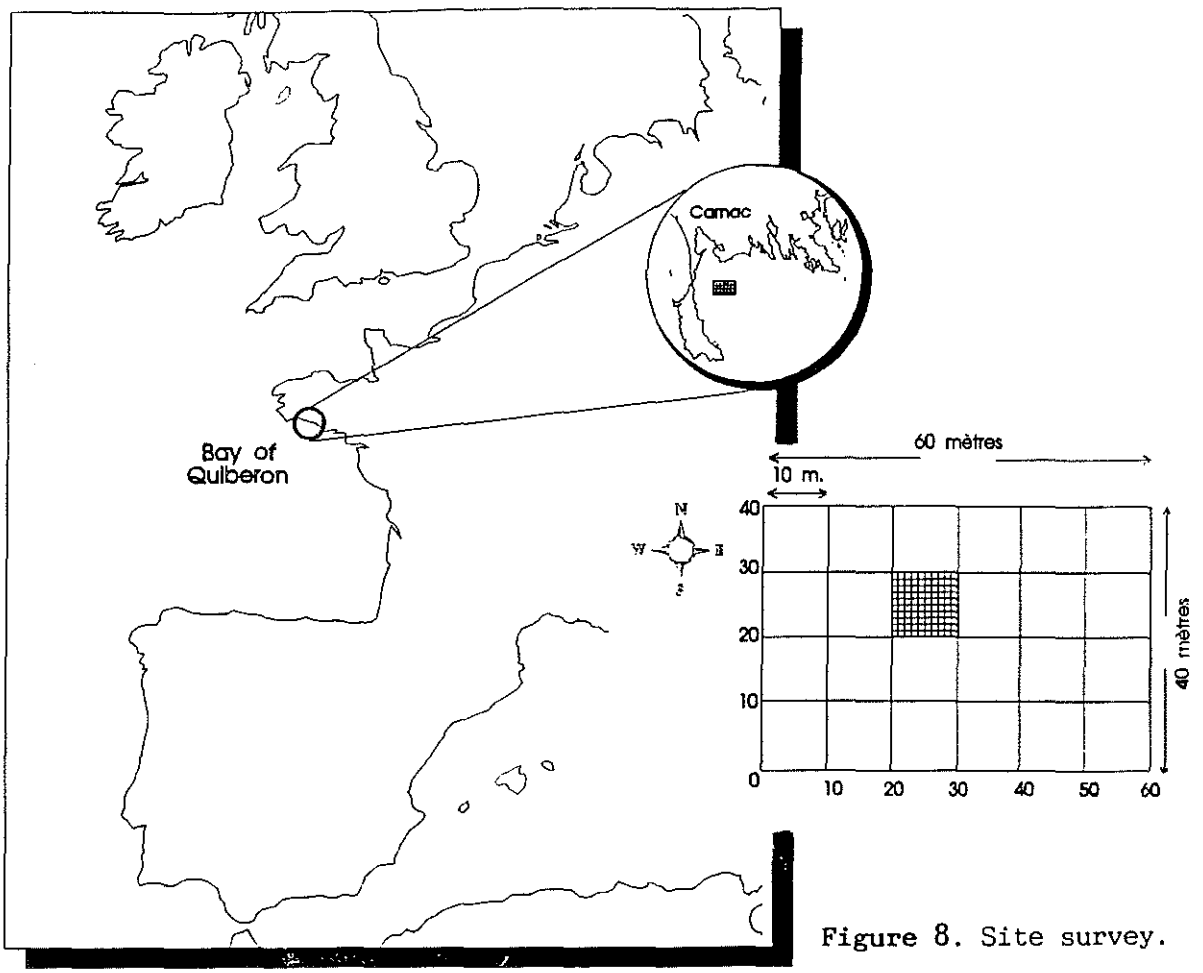
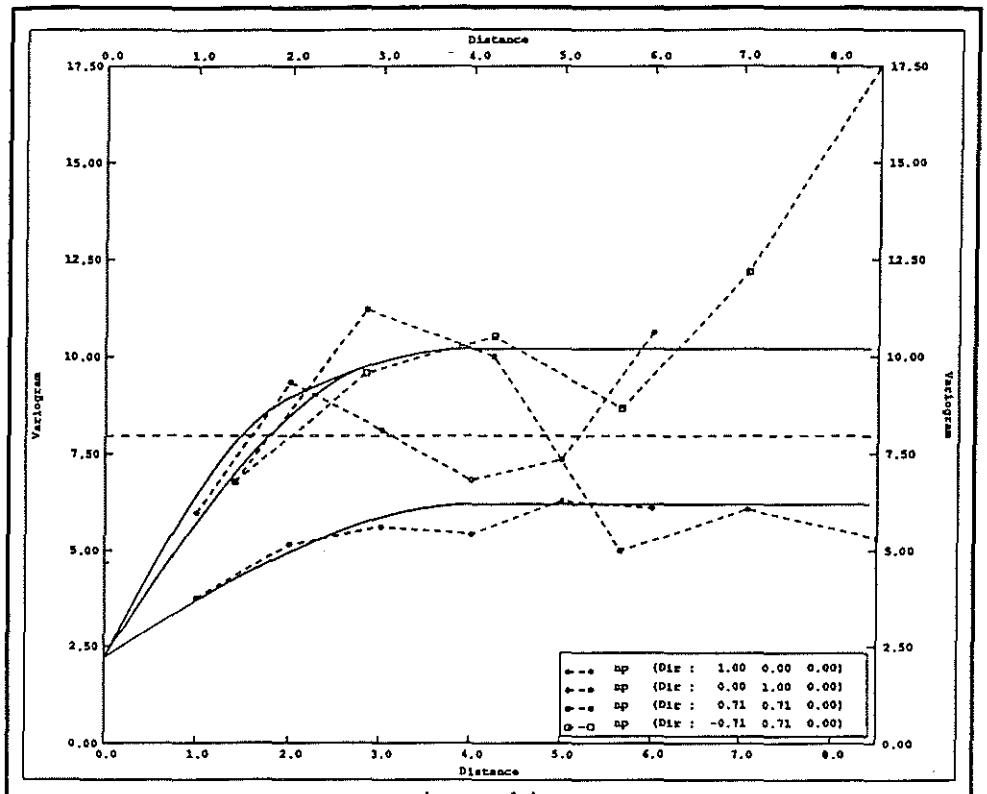


Figure 8. Site survey.



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NUGGET:	2.20 NB. STRUCTURE(S):2			ANISOTROPY		
STRUCTURE	SILL	RANGE	LAMBDA	X	Y	Z
spheric.	4.000	4.000		1.000	1.000	1.000
spheric.	4.000	2.000		0.000	1.000	1.000

Figure 9. Modelisation of the variogram of the number of parasited oysters per square meter.

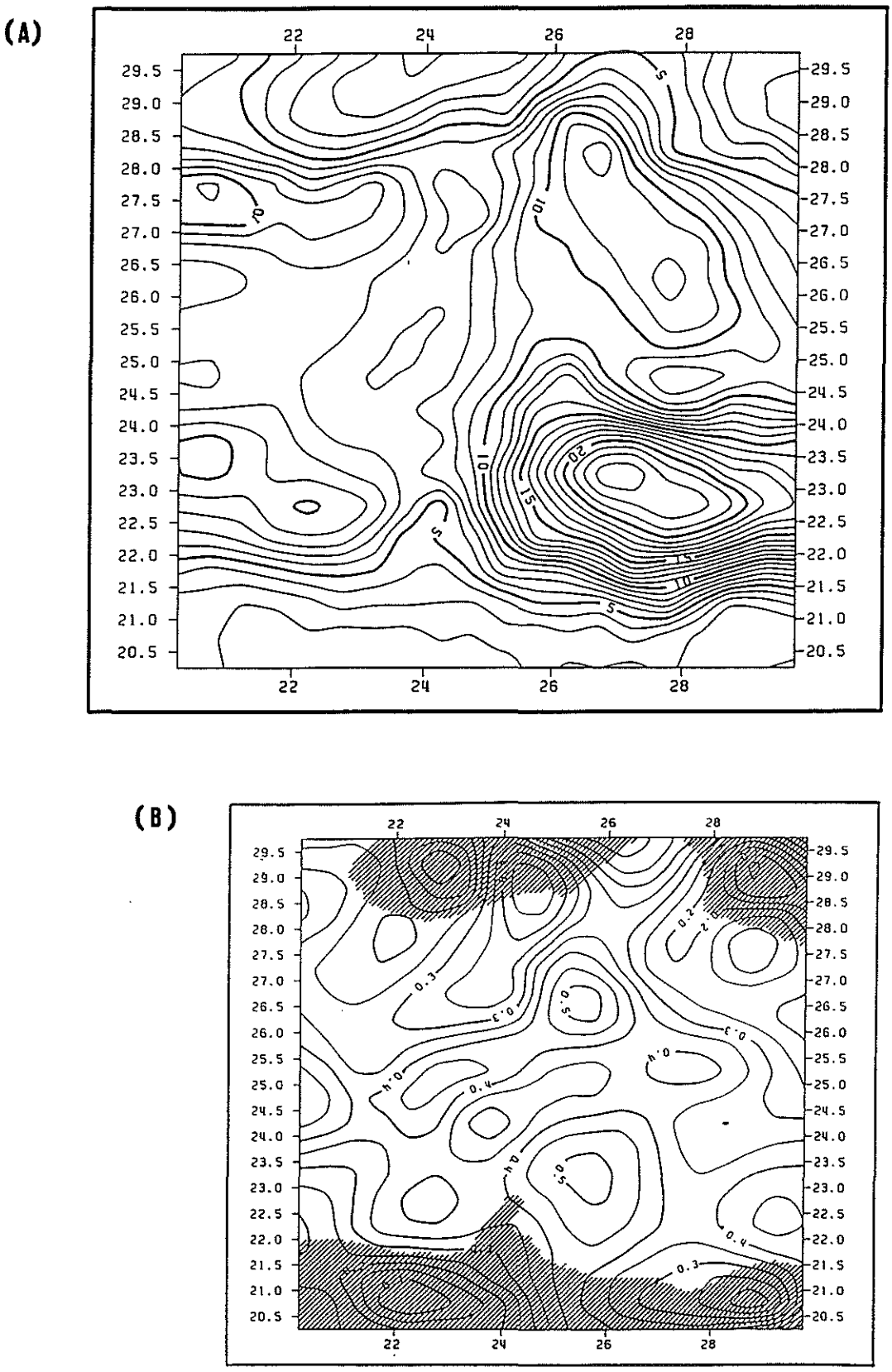


Figure 10. Distribution map of oyster density (A) and of *Bonamia* infested oyster proportion (B) at small scale (10m x 10m) (0.3 = 30 percent)

PART II

ANALYTICAL EPIDEMIOLOGY

N. COCHENNEC, S. CULLOTY, J.MAZURIE, J. MONTES and M. MULCAHY

CONTENTS

CHAPTER 1

INTRODUCTION

In order to control and to be able to reduce the impact of the bonamiasis in the different rearing areas, it appeared necessary to study and understand the role of environmental and zootechnic parameters which can be involved in the development of *Bonamia*. Information in the litterature on patterns of infection indicate that infections occur throughout the year, but highest prevalence seems occur in the summer, others in the autumn (7,8,9). Environmental factors including temperature and salinity do not appear to play a major part of the transmission of the disease as bonamiasis has been found at temperature as low as 4°C. Moreover, the age would be related to differences in susceptibility with older oysters appearing to be more susceptible (7,10).

In this study, some parameters have been considered and mostly, the influence of the age of the oysters and the season, the influence of some zootechnic parameters such as the depth of the oyster culture and the transfer operations of the oysters.

Finally, attention has been paid to the understanding of the development of the disease at the animal level. Study of the parasite *B. ostreae* has been hampered because of lack of information on its life cycle. It appears that transmission can occur from oyster to oyster. The availability of purified parasites (11) has made it possible for the disease to be experimentaly reproduced by inoculation of parasite into oysters (12). However, the early events of infection are unknown. This stage corresponds to a latent period during which the location and fate of *B. ostreae* has not yet been determined.

On the other hand, it has been assumed that the parasite first has an infective stage in the ovarian tissues of the oyster before the haemocytic stage which develops as a result of phagocytosis of residual gametes (7). So, the study of the relation between the oyster gametogenesis and the presence or absence of the parasite was undertaken on the basis of monthly histological analysis for prevalence and sex determination.

CHAPTER 2

MATERIALS AND METHODS

2.1 INFLUENCE OF SEASON AND AGE OF THE OYSTERS

2.1.1 Oysters

Oysters used in this study were of two age classes, 1987 and 1988 stocks. Both groups were being ongrown in the North Channel of Cork harbour, in Ireland, at densities considered to be extremely low by commercial standards. Depth varied from 2-8 feet, salinity from 28-30% and temperature fluctuated from 8°-10°C in winter and 16°-18°C in summer. The 1988 group were monitored monthly and the 1987 group every second month from February 1990. In samples, the count of dead oysters was taken and sub-samples of 58 live animals was made.

2.1.2 Diagnosis of *B. ostreae*

Bonamia infection was determined by examination of heart smears. The ventricule of the heart was removed, dabbed onto tissue to remove excess moisture and then dabbed onto a dry slide to prepare haemolymph smears. Slides were fixed in methanol for approximately 40 sec and then stained with Hemacolor rapid blood smear (Merck).

2.2 INFLUENCE OF DEPTH

Three populations of flat oysters (*Ostrea edulis*) from Spain areas, Fornelos (F population), chonically infected area, San Ciprian (C population), a *Bonamia*-free area and Norway (N population), were cultured in the Arosa estuary at two different depths (1-2 and 8-9m) to test influence of the oyster origin and the depth of the culture on the development of bonamiasis.

2.3 INFLUENCE OF OYSTER TRANSFERS

Two batches of 200 kgs of 2 year old flat oyster (spawning 1986) from the St Michel Bay (France) were transfered in Quiberon Bay: one batch during March 89 and the other during May 89. Each of them were released within an area of 10 m^2.

The infection rates were controled by histological examination of sections or by ELISA test (see methods PART I-CHAPTER 2).

2.4 RELATIONSHIP BETWEEN GAMETOGENESIS AND BONAMIASIS

2.4.1 Oysters

Flat oysters originating from Quiberon Bay (France) were reared in two different areas, the Paimpol Bay in North Brittany and Quiberon. Samplings were made monthly from May 1990 to December 1990. Complementary studies, in Ireland examined 2 year old oyster stock, monitored over 2 year and 3 year old oysters, over one year.

2.4.2 Diagnosis of the disease and sexing of oysters

The infection rates were established on histological sections and smears as previously described.

The sexing of oysters were determined on gonad smears. A small incision was made in the gonad and a clear dry slide was pressed against it. The smear was allowed to dry, then stained with "Hemacolor kit" (Merck) and examined for the presence of spermatozoa or oocytes. The gonad development stages were determinated by examination of histological sections of gonads.

2.5 STUDY OF THE LATENT PERIOD OF INFECTION

2.5.1 Oysters

Oysters from Tralee Bay (Ireland) were stabilised in the laboratory for six weeks prior to inoculation. They were held in aerated seawater, and fed daily on *Tetraselmis sueccia*. These disease-free oysters have been experimentally inoculated with *Bonamia*. Control oysters were inoculated with filtered seawater. Oysters from both control (n=3) and experimental group (n=10) were sacrificed 2, 5, 9, 14, 21, 28, 35 and 42 days post-inoculation.

2.5.2 Diagnosis of *B. ostreae*

Heart smears were made and tissues were fixed and embedded in paraffin for histology. Serial sections through the tissues were cut and examined after staining.

Indirect immunofluorescence assay were carried out on sections, using a *Bonamia* specific monoclonal antibody (Sanofi) counterstained with a FITC-conjugated mouse immunoglobulin antiserum (Pasteur).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 INFLUENCE OF ZOOTECHNICAL PARAMETERS AND SEASON ON BONAMIASIS

3.1.1 Depth

The bonamiasis developed faster in the population (F) from Fornelos than in those from San Ciprian (C), but after 15 months, the population F presented a high mortality (88%). The low prevalences of *B.ostreae* in the population C can be explained by the age of the oyster stock, which was composed of young animals. At the end of the experiment, the mortality in this population reached 42% when reared at 8 m depth.

Bonamia ostreae was first detected and with higher prevalences in the oyster populations reared at the depth of 8-9 m compared to those reared at 1-2 m (table 3). The death rates appeared higher in the oysters cultured at 8-9 m. Nevertheless, the infection rates were not significatively different (Chi squared test, P= 0.05) between the oyster reared at the two depths. Given these results it would seen advisable for raft-cultured oysters to be reared at a depth of 1-2 m.

		Infection rate				
BATCH	DEPTH	MARCH 90	JULY 90	OCT 90	JAN 91	JULY 91
N	1-2 m	0	0	?	10%	28%
	8-9 m	0	?	?	20%	35%
F	1-2 m	0	0	13%	13%	23%
	8-9 m	0	13%	17%	27%	30%
C	1-2 m	0	0	0	0	3%
	8-9 m	0	0	0	3%	16%

Table 3. - Evolution of the *Bonamia* infection in each oyster population of Galicia (Spain).

N: Norway population; F: Fornelos; C: San Ciprian.

3.1.2 Transfer

As stock movements such as relaying and transfers appear to increase the susceptibility of the oysters to *B. ostreae*, they have been inadvisable ever since the outbreak of the disease in France. However, some trials have been carried out: transfer for fattening, broodstock transfers or 18 month old oysters released after fishing and sorting. These operations have led

N°	SETTLEMENT DATE	TRANSFER DATE	TRANSFER CONDITIONS	INITIAL PREVALENCE %	1st CONTROL DATE	PREVALENCE %	PREVALENCE OF BATCH SAME AGE Origine area	Rearing area	2nd CONTROL DATE	PREVALENCE %
1	Settlement 1988	February 1991	Fishing Transport Sorting Seeding	3/50	May 1991	7/52 13,5%	0/101 0%	54/76 71%	September 1991	19/99 19%
2	Over settlement 1989			6%		5/28 17,9%	0 à 2/50 0 à 4%	0/50 2/95 0 à 2%		
3	Settlement 1987	Winter 1989 - 1990	Fishing Sorting Seeding	2/100 2%	May 1990	5/32 15,6%	0/50 0%	/	June 1990	19/30 63%

Table 4. - Evolution of *B. ostreae* after the transfer of flat oyster batches, 2 and 3 years old.

1 and 2 : Cancale Bay (deep water) ----→ St-Brieuc Bay (deep water)

3 : Morlaix Bay (deep water) ----→ Morlaix Bay (deep water)

to a fast increase of the infections but the respective influence of the different parameters involved have not been determined (table 4). The effect of the period of transfer was studied in detail.

The initial infection rates of the two transfered batches of oysters were respectively of 1% and 3% in March and May 1989. Seven months after the first transfer, histological analysis and ELISA tests were performed which showed a significant increase of the infection rates (P=0.05)(Table 5). The batches transfered in March appeared less infected (5%) than those transfered two months later (16%). At the same time, the batch of oyster reared in Cancale Bay without any transfer or manipulation was comparable in terms of infection with the batch transfered in March (5%). However, this difference disappeared after 15 months of rearing (26% instead of 20%).

So it would seen that a early transfer can be made but the subsequent fishing out of the broodstock after spawning remains a necessity.

Date	Cancale batch in situ	transfered batch in march 1989	Comparison test	transfered batch in may 1984
14/03/89	1/100	1/100	/	/
18/05/89	2/100	3/100	non sign.	2/100
10/10/89 23/10/89	6/100	5/96	sign. p = 0.01	14/88
06/06/90	/	26/100	non sign.	20/94

Table 5: Transfer assay on flat oysters at two period of the year: evolution of *B. ostreae* infections.

3.1.3 Seasonal variation in bonamiasis

The natural seasonal variation of infection and mortality due to *B. ostreae* have been monitored on oysters from beds in the North Channel of Cork harbour in Ireland. The oyster densities are considered to be extremely low by commercial standards. Two groups were considered, oysters spawned in 1987 and in 1988.

In Cork harbour, the temperature fluctuates from 8°-10°C in winter to 16°-18°C in summer. The table 6 and the figures 11 and 12 would indicate that much variation in prevalence of infection occurred from month to month in both age groups. A look at the seasonal component would indicate that peaks of infectivity occurred in the 1988 group in January and April (fig.11) and in the 1987 group, levels appeared to remain high in the first half of the year to July (fig.12). There appears to be a continual cycle of infectivity and mortality occuring on the beds. Chi-square tests carried out for both 1987 and 1988 groups indicated that there was a very significant association between infection levels and sampling date ($P<0.0001$).

	1988 stock			1987 stock		
Month	AGE	% INFECTED	% MORTALITY	AGE	% INFECTED	% MORTALITY
Feb.'90	1.5	0.0	8.50	2.5	36.40	-
May	"	-	-	"	79.70	-
June	"	0.0	-	"	32.80	10.00
July	2.0	-	-	3.0	57.90	-
Aug.	"	1.70	58.00	"	-	-
Sept	"	31.00	-	"	-	-
Oct.	"	12.10	-	"	-	-
Nov.	"	35.10	-	"	-	-
Dec.	"	37.90	-	"	-	-
Jan.'91	2.5	65.50	56.00	3.5	68.4	-
Feb.	"	29.30	8.00	"	67.90	-
Mar.	"	33.30	8.00	"	-	-
Apr.	"	62.10	8.00	"	-	-
May	"	29.30	-	"	72.40	-
June	"	-	-	"	-	-
July	3.0	17.40	21.30	4.0	50.00	64.30
Aug.	"	29.30	22.70	"	-	-
Sept.	"	19.60	37.00	"	18.90	39.30
Oct.	"	35.70	14.60	"	-	-
Nov.	"	9.62	12.38	"	17.65	45.67
Dec.	"	33.33	19.75	"	-	-
Jan.'92	3.5	36.21	22.07	4.5	41.38	21.38

Table 6. - Seasonal variation in prevalence of infection and % mortality over 24 months.

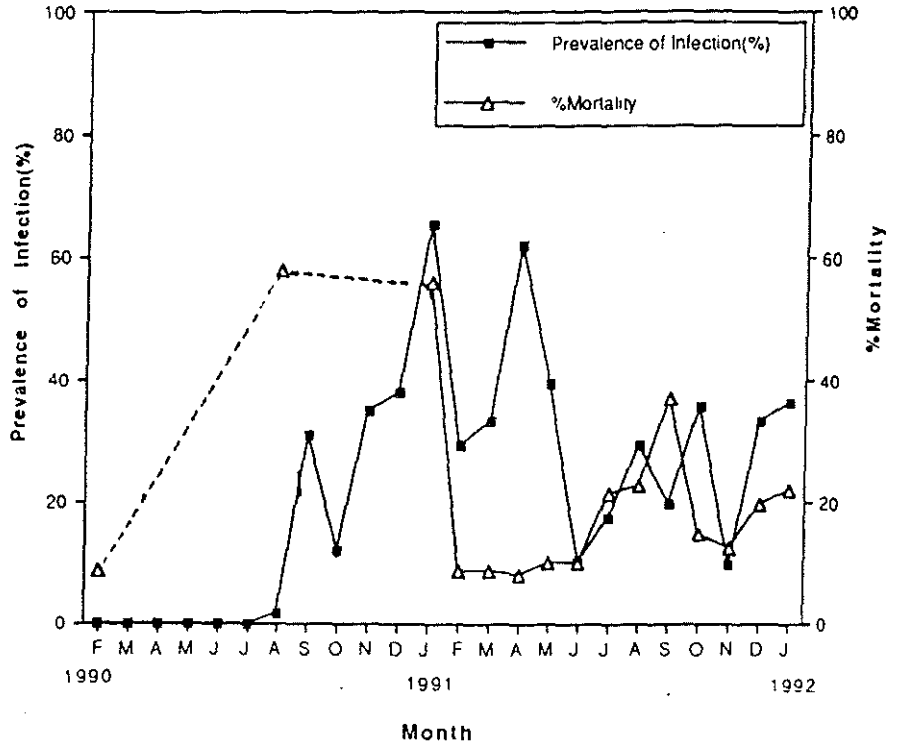


Figure 11. Variation in prevalence of infection (%) and % mortality in the 1988 group from Feb.1990-January 1992. (... no intermediate results available and data extrapolated).

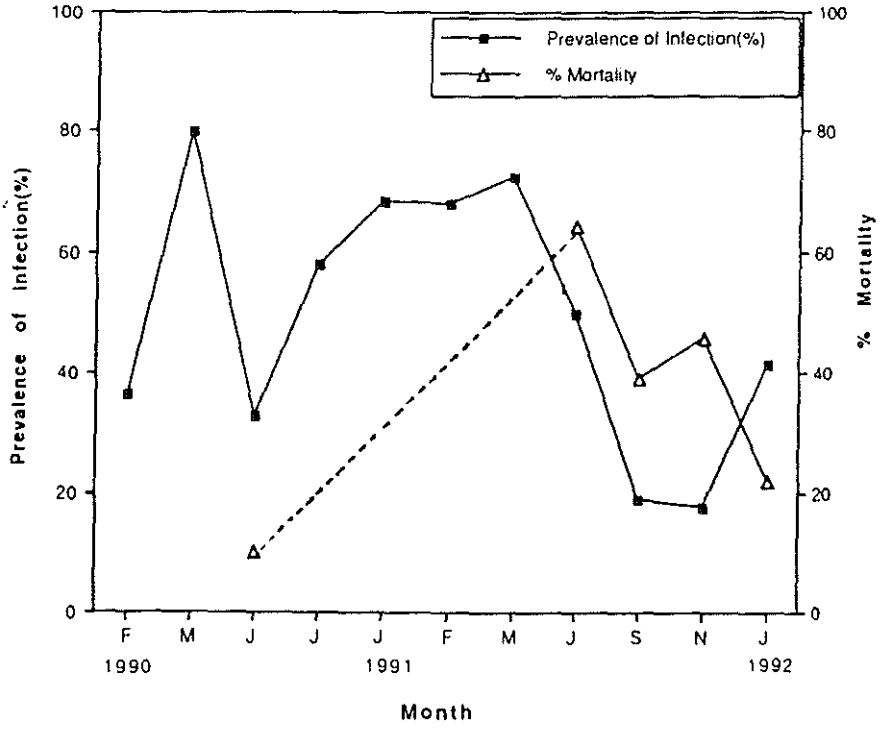


Figure 12. Variation in prevalence of infection (%) and % mortality in the 1987 group from Feb.1990-January 1992. (... no intermediate rsults available and data extrapolated).

The initial diagnosis of infection in the 1988 group was made in August 1990 when the oysters were approximately 2 year old (Table 1). A large increase to 31% prevalence was noted in September and levels continued to rise in the following months peaking in January 1991 at 65.5%. Mortalities in this month were also high at 56%. Throughout the spring months prevalence remained high, another peak of 62.1% being recorded in April. Mortalities during these months remained low at approximately 8%. A drop to 17.4% infection occured in June 1991 which was associated with a slight increase in % mortality to 21.3%. Prevalence of infection and mortalities continued to fluctuate over the autumn of 1991. In November prevalence and mortality dropped to 19.6% and 12.4% respectively. January 1992 did not show the large increment in infection and mortality that had been observed twelve months previously.

The 1987 group were already infected at the start of the period of observations when the oysters were aged 2.5 years (table 6). In this group a peak of prevalence of infection occured in May 1990 at 79.9% and is followed in June by a large drop to 32.8%, accompanied by a mortality level of 10%. An increment in prevalence was observed in July at 57.9% but a further examination of this group did not occur until January 1991 when 68.4% prevalence was observed, a result similar to that obtained for the 1988 group the same month. The 1987 group showed a fluctuation in levels in the autumn of 1991 similar to that observed in the 1988 group.

The heaviest intensities of infection occurred in the 1988 group in November 1990, and January and April 1991, having class ++++ infections of 8.8%, 7.3% and 6.9% respectively. In all other months, class ++++ infections varied from 0-3.6% (fig.13). In the 1987 group, 27.7% of oysters in May 1990 had a class ++++ level of infection, 14% and 15.5% in July 1990 and July 1991 respectively. Apart from three peaks class ++++ infections varied from 0-67.1% (fig.14).

Examination of prevalence of infection in both age groups would indicate that levels vary widely in both groups. Initial results in February 1990 indicated that at 18 months approximately the 1988 group were still uninfected even though having been on infected beds for over a year and in Cork harbour water throughout their lives. The 1987 group which at this stage were approximately 30 months showed very variable prevalence of infection over these months up to 79.7%. Once the 1988 group picked up the infection in August 1990, infection levels rose dramatically. Both groups showed the same levels of infection on different months. When comparing the prevalence of infection of the two groups at the same age, no similarity exists (table 7). Data for four months is available for this comparison e.g. when the 1987 stock were approximately 3 years old, 57% were infected in July 1990 compared to 17.4% infected in the 1988 stock at the same age.

The influence of interbed variation in this experiment is unknown. Monitoring of these stocks was confined to two beds for logistic reasons - it has been noted that infection levels on different beds can differ significantly.

The onset of observable infection in the 1988 group at 2 years of age agrees well with previous studies. The sharp increase in levels in the following months may be associated with post-spawning stress and high levels mid-winter may be related to a stressor such as low food availability. Lower prevalence of infection and mortalities in the two groups in the autumn of 1991 may be a result of reduced stocking density

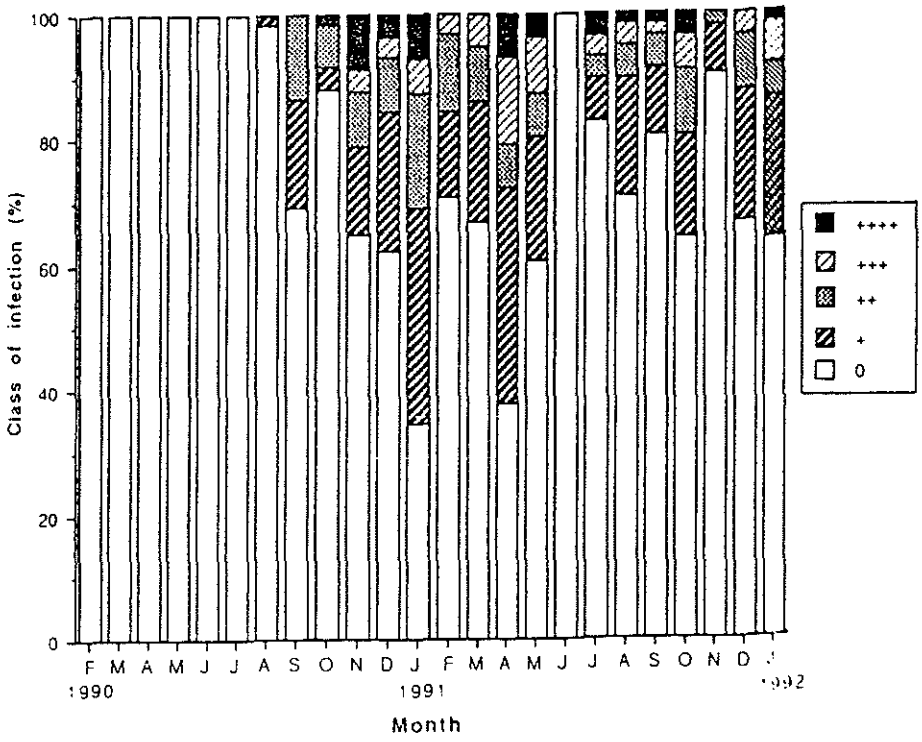


Figure 13. Seasonal variation in intensity of infections in the 1988 group.

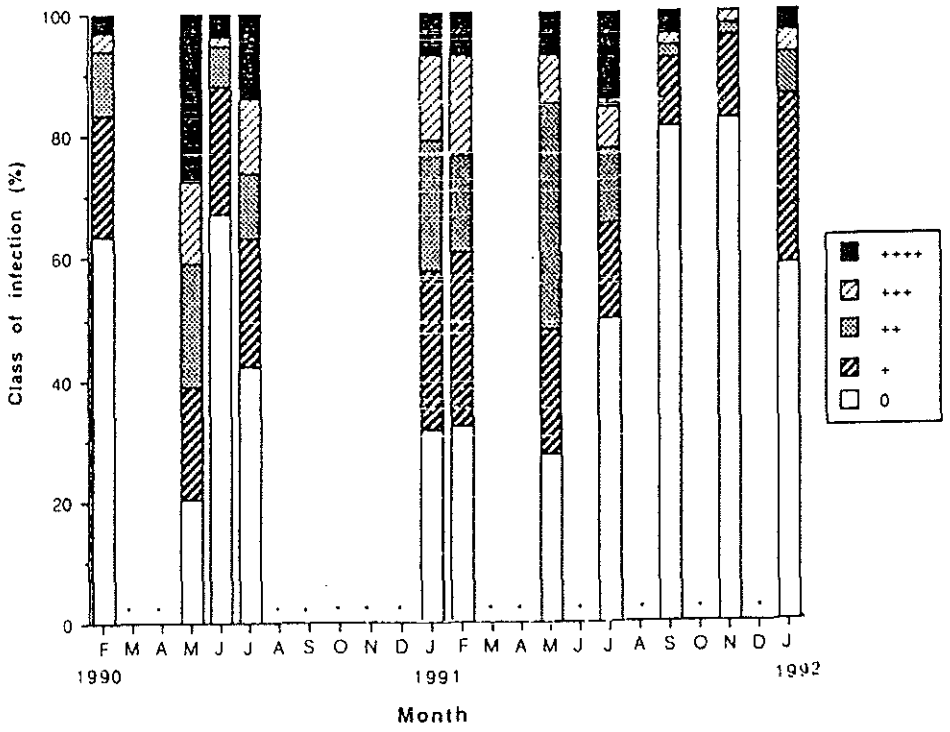


Figure 14. Seasonal variation in intensity of infections in the 1987 group. (* oysters not sampled on these months).

	1988 stock			1987 stock		
AGE	MONTH	% INFECTED	% MORTALITY	MONTH	%INFECTED	%MORTALITY
1.5	Feb.'90	0.00	8.50			
"	Mar.	0.00	-			
"	Apr.	0.00	-			
"	May	0.00	-			
"	June	0.00	-			
2.0	July	-	-			
"	Aug.	1.70	58.00			
"	Sept.	31.00	-			
"	Oct.	12.10	-			
"	Nov.	35.10	-			
"	Dec.	37.90	-			
2.5	Jan '91	65.50	56.00			
"	Feb.	29.30	8.00	Feb. '90	36.40	
"	Mar.	33.30	8.00	Mar.	-	
"	Apr.	62.10	8.00	Apr.	-	
"	May	29.30	-	May	79.70	
"	June	-	-	June	32.80	10.00
3.0	July	17.40	21.30	July	57.90	
"	Aug.	29.30	22.70	Aug.	-	
"	Sept.	19.60	37.00	Sept.	-	
"	Oct.	35.70	14.60	Oct.		
"	Nov.	9.62	12.38	Nov		
"	Dec.	33.33	19.75	Dec.		
3.5	Jan.'92	36.21	22.07	Jan.'91	68.40	
"				Feb.	67.90	
"				May	72.40	
4.0				July	50.00	64.30
"				Sept.	18.90	39.00
"				Nov.	17.65	45.67
4.5				Jan '92	41.38	21.88

Table 7. - Comparison of prevalence of infection and % mortality in 1987 and 1988 stock at the same age.

due to harvesting in the 1987 group and to mortalities which occured in the previous 12 months.

3.2 STUDY OF THE DEVELOPMENT OF THE DISEASE

3.2.1 Relationship with gametogenesis

Two aspects of the relationship between disease prevalence and the sex of the gonads have been examined. Firstly, to determine the effect of the bonamiasis on the oyster reproduction and secondly, to determine if the initial focus of infection was in the gonads.

Firstly, 684 flat oysters from two rearing areas of Brittany (Quiberon and Paimpol) were examined during the maturation period of May to December 1990. The infection rates were compared to the development of the gonads. Five maturation stages were distinguished on the basis of examination of histological sections of gonads:

I : Indifferentiated gonad
M : Male
F : Female
ML: Male after laying
FL: Female after laying

For each animal, increasing infection rates were estimated as 0, +, ++ and +++. Three age groups have been also considered for the oysters in each area: Quiberon 1 : 1 year old oysters, Quiberon 2 and Paimpol 2 : 2 years old and Quiberon 3 : 3 years old oysters.

As shown in the figure 15, regardless of the area, after a period with a lack of differentiation, the majority of the oysters displayed at least two maturation cycles. The sex-ratio points to a male predominance at the beginning of the spawning period and an incease of the females at the end of the year. During 1990, two spawnings occurred. The oysters of different ages matured and spawned at different times.

During the study, an increase of the infection rates was noticed from July 1990 affecting particularly the females and the undifferentiated oysters, more numerous at this period (figures 16 and 17).

The maturation and the development of the infestations seemed comparable in Quiberon and Paimpol areas. The presence of *B. ostreae* did not appear to inhibit the gametogenesis. However, the stress due to the laying could accelerate the development of the infections.

Van Banning (7) points to the initial focus of *B. ostreae* as being the female follicles with infections being picked up by the hemocytes when phagocytosis of residual gametes occurs.

In order to verify this hypothesis, experiments were carried out by examination of two oyster stocks, 1987 and 1988, respectively 2 years and 3 years old, from Cork harbour in Ireland. Over two years, the gametogenesis have been studied on gonad smears and compared to infection rates. Male and female gonads were easily distinguishable under low power following smearing onto slides and staining. The percentage of males and females infected was calculated (results are in brackets in table 8a and b). In the 1988 group from May 1990 to May 1991, female oysters were uninfected and in July 1991 had a lower prevalence than male oysters. However, in August 1991,it appears that a similar percentage of the females compared to males were infected, i.e. 29.6% of the males who composed 75% of the population compared to 30.8% of the females who composed 22.4% of the population. A similar situation existed in the 1987 group with female oysters having a lower prevalence of infection than males in July 1990 and 1991 (table 8b).

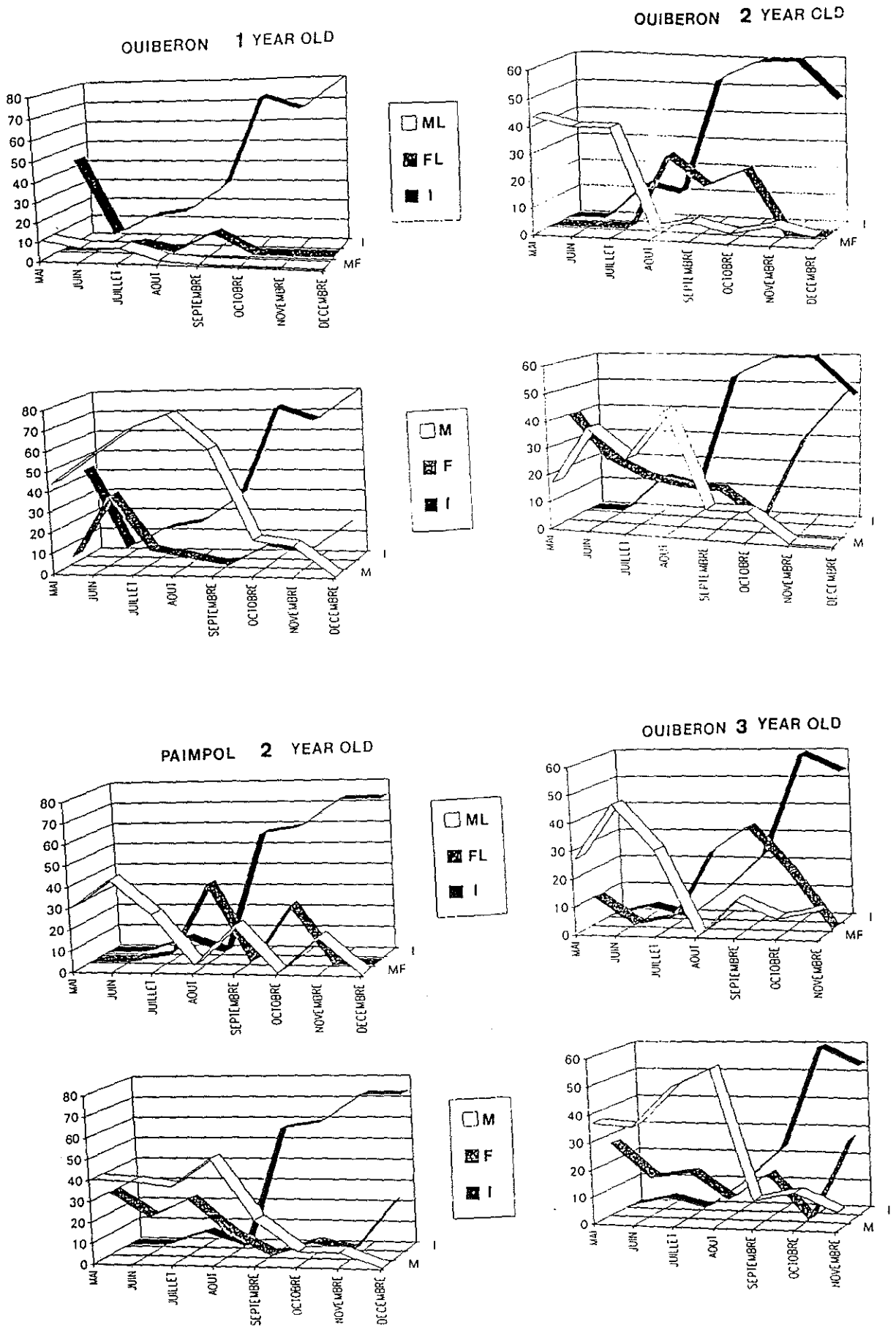


Figure 15. Development of the gonad during the maturation period of May to December in different oyster age groups and areas

I : indifferentiated gonad
M : male
F : female
ML : male after laying
FL : female after laying.

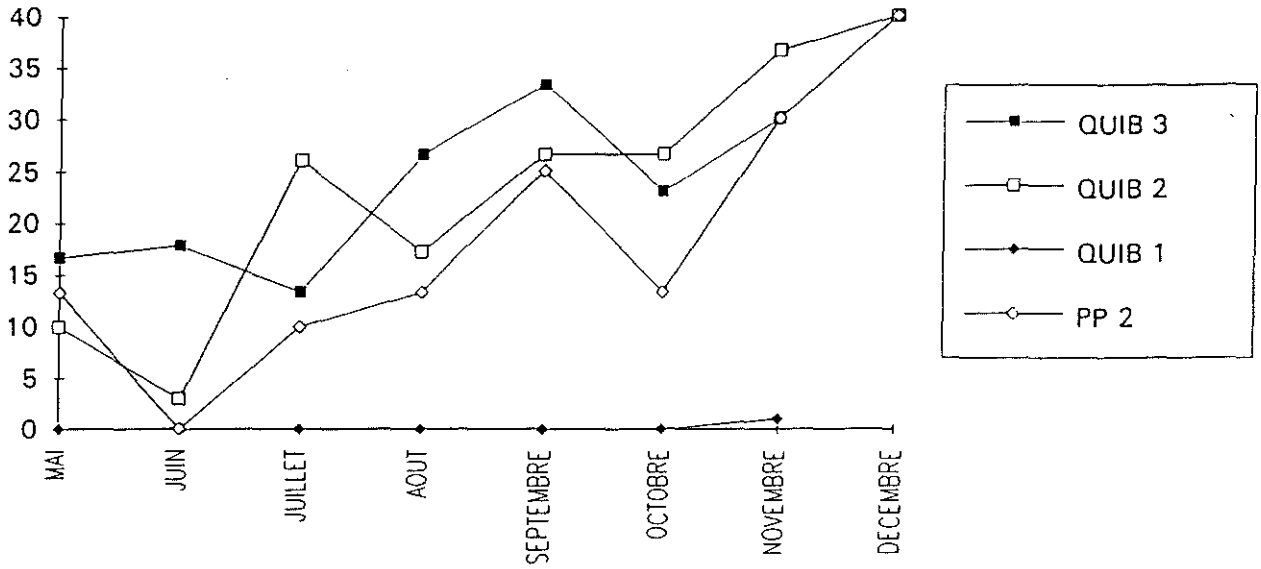


Figure 16 : Infection rates of *B. ostreae* during the period of May to December in different oyster classes of age.

I : indifferentiated gonad
M : male
F : female
ML : male after laying
FL : female after laying.

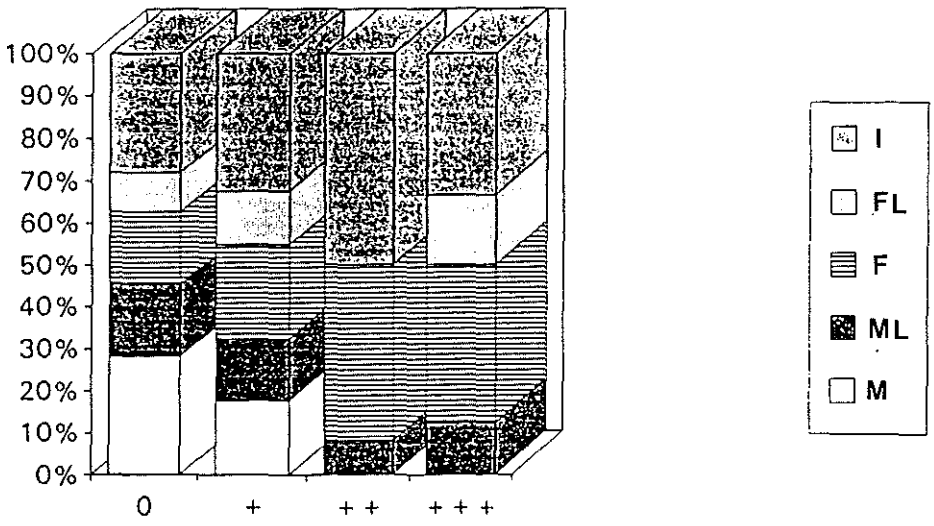


Figure 17. Comparison between several maturation and infection rates of *B. ostreae*.

QUIB 3 : Quiberon 3 years old oysters
QUIB 2 : Quiberon 2 years old oysters
QUIB 1 : Quiberon 1 year old oysters
PP 2 : Paimpol 2 years old oysters

a - 1988 stock

MONTH	% MALE (% infected)	% FEMALE (% infected)	%MALE/FEMALE (% infected)	? Sex (% infected)
June 1990	80.00	20.00		
	(0.00)	(0.00)		
August	94.00	6.00		
	(7.50)	(0.00)		
January '91	100.00	0.00		
	(62.96)	(0.00)		
February	100.00	0.00		
	(25.00)	(0.00)		
March	93.10			6.90
	(31.48)			(50.00)
April	54.7			45.30
	(51.72)			(68.00)
May	78.95	10.53		10.53
	(13.33)	(0.00)		(0.00)
July	68.75	25.00		6.25
	(24.24)	(16.67)		(33.33)
August	75.90	22.40	1.70	
	(29.55)	(30.77)	(0.00)	

b - 1987 stock

MONTH (% infected)	% MALE (% infected)	% FEMALE (% infected)	% MALE/FEMALE (% infected)	? Sex
July '90	75.00	25.00		
	(62.50)	(38.46)		
Feb. '91	100.00			
	(66.04)			
May	100.00			
	(77.50)			
July	75.50	14.30	10.20	
	(54.05)	(28.57)	(40.00)	

Table 8. - Sex ratio of 1988 stock (a) and 1987 stock (b) obtained from gonad smears. % of each sex infected in brackets.

In the summer months of 1990, 80-94% of the population were male and a mean of 75.0% (5.2, standard deviation) were male in the following summer. During winter and early spring 1991, 100% of the gonad smears showed male gamets (table 8a). Data for the 1987 group is limited but indicates similar levels - 75% males in July 1990 and 1991 and 100% males in February and May 1991 (table 8b).

The results obtained here would not strongly support van Bannings'hypothesis. However it must be acknowledged that the smear technique is indicative rather than absolute. Pure females without developing eggs are known to occur as are ripe females without male gametes. Histological section analysis are therefore more appropriate for such a study.

3.2.2 Study of latent period of the infection of *B. ostreae*

The events of the early phases of infection of *B. ostreae* remain unknown. The initial location of the parasites in its host tissues has not yet been determined, corresponding to a latent period of infection of several weeks. This period was observed to least for 6-8 weeks, on the basis of experimental infections in which, disease-free oysters were inoculated with purified parasites and sacrificed over a range of post-inoculation intervals of time. Samples were examined by heart smears and serial sections of tissues, after staining or after reacting with a specific monoclonal antibody in an indirect immunofluorescent assay. The presence and location of *B. ostreae* or any potential *Bonamia*-like structure was recorded together with any host response.

2 days post-inoculation:

Bonamia cells were observed in 2 out of 10 oysters in the connective tissue, presumably being part of the inoculate.
Exceptional infiltration of haemocytes throughout the connective tissue was observed in one of the oysters. No *Bonamia* was seen on heart smears.

5 days post-inoculation:

Single *Bonamia* cells were observed extracellularly in 5 oysters in the epithelium of the digestive diverticulum, the gills, adductor muscle and connective tissue. One parasite was observed intracellularly in a haemocyte.
5 out of 11 oysters had exceptional infiltration of haemocytes throughout the tissues.
No heart smears showed *Bonamia* cells.

9 days post-inoculation:

One parasite was observed extracellularly near the gonads and in another oyster, a single Bonamia was extracellularly in the connective tissue. 7 ou of 10 oysters showed infiltration of haemocytes. Heart smears and non inoculated controls were again clear.

14 days post-inoculation:

Bonamia cells were observed extracellularly in 2 oysters in a sinus and in the gonads and another oyster had 2 parasites intracellularly in a haemocyte and the intestinal epithelium.
Again, 5 out of 10 oysters showed inflammatory reactions.

21 days post-inoculation:

An intracellular form of *Bonamia* was observed in a haemocyte. 8 out of 10 oysters showed infiltration throughout the tissues.
No parasites was identified in heart smears.

28 days post-inoculation:

B. ostreae cells were observed in the adductor muscle and many were observed in one oyster in the mantle epithelium. Parasites were observed

intracellularly in haemocytes in the connective tissue and in the gonads in 2 oysters. All 10 oysters showed infiltration by haemocytes. For the first time, 2 heart smears showed infection, one a class + and the other a class ++ infection.

35 days post-infection:

No parasites were observed. All 10 oysters presented inflammatory reactions. The heart smear of one oyster showed a class + infection.

42 days post-infection:

All oysters were infected. *Bonamia* cells were observed in all tissues both intra- and extracellularly. Exceptionally heavy haemocyte infiltration was visible in all experimentally infected oysters. All heart smears showed classes of infection from + to ++++. All controls were uninfected.

B. ostreae was sparsely distributed, in inoculated oysters over the first 35 days. The observations suggest that the parasites which were inoculated were dispersed through the tissues but no concentration of parasites were observed. From day 28 onwards *Bonamia* was observed in some heart smears. However, by day 42 widespread *Bonamia* was observed in tissues and heart smears which implies that multiplication of parasites from the original inoculate had occured.

Detailed screening of tissue sections during the period 0-35 days for possible intermediate stages of *B. ostreae* revealed only a small number of uni- and binucleated cells, located in the epithelium of the stomach, only at day 5 and day 9. These forms were not specifically recognized by the anti-*Bonamia* monoclonal antibody used in indirect immunofluorescent assay, when the parasites were strongly positive. The significance of these cells is unknown but they could correspond to algal cells present in the gut.

Infiltration of all tissues by haemocytes was progressively observed from day 2 onwards in oysters inoculated with *Bonamia* but not in controls.

CHAPTER 4

CONCLUSION

Oysters infected with *B. ostreae* were found throughout the year as found in other studies (8,9). Very significant variations in infection levels were shown from month to month in Irish oyster stocks but no obvious seasonal pattern of infection and mortality was present. The results would indicate that heaviest infections and mortalities occurred from mid-winter through to early summer unlike other studies where highest mortalities and infections occurred from early spring to autumn (7,9). The different observations from different countries would indicate that all stressor conditions linked to unfavourable environmental parameters such T° as food shortages, may exacerbate infections, the increase stress in the animals resulting in higher mortalities during this period. Obviously the parasite is present and transmissable throughout the year and it appears that a continual cycle of infection and mortality occurs.
It has been noted that oysters up to 18 months generally have very low infections and begin to show heavy infections at around two years of age. Mortalities can be low until the oysters are 24 months old and after that they reached higher values (13). Different investigations have indicated that older oysters appear more susceptible to infection (10,14,15) or show heavier intensity of infection. This may be caused by post-spawning stress, but also to a longer time being available for the build-up of infection or higher stocking density. As shown in this study, comparisons infections at the same age showed no similarity in prevalence of infection indicating that age alone does not determine infection.

It appeared from the general results that zootechnical parameters such as depth of culture and stressors such as oyster handling and transfers, would lead to a significative increase of the susceptibility of the oysters and spreading of the disease. Oyster transfers can be made early in the year, before the maturation period and the removal of broodstock must be done immediately after the breeding period in order to avoid a significant increase of infection.

In order to be able to establish effective zoosanitary prophylactic measures, it is necessary to locate the initial infection focus of *Bonamia* and the development kinetics of the disease.

It is generally considered that the transmission of the parasite occurs horizontally. Van Banning (7) pointed to the initial focus of *Bonamia* as being female follicles, with initial infections occurring after the female phase when phagocytosis of residual gametes occurs, leading to the the haemocytic phase. In fact, such hypothesis was not verified in this study when the relationship between *Bonamia* prevalence and the oyster sex was examined in either French, or Irish breeding areas. Both males and females were affected by the parasite. In different European countries, it was found that the flat oyster is an alternating hermaphrodite with young oysters first maturing as males. After about two years, when heavier infection rates often occur, most oysters function both as males and females at least once a year.

If it is assumed that the infection develops in haemocytes, the exact location of early infection stage remains unknown. With the establishment of experimental reproduction of the bonamiasis, whose

applications are presented in Part IV, the study of the development kinetic of the disease have been undertaken.

No cryptic forms of the parasite or other stages in the life cycle were discovered despite extensive examination of sections. In the days following inoculation, all that was observed were single *B. ostreae* that were presumed to be part of the inoculate, in which each oyster received an infective dose of $5x10^5$ parasites. However, the small size of the cells may have been a factor, given the limited of sensitivity of visual examination of sections. On the other hand, the parasites may have been eliminated from the tissues. In the days following inoculation small numbers of haemocytes were observed in the connective tissue of oysters, some individuals had dense aggregates. *Bonamia* cells were generally not observed in these aggregates, but this would suggest a defence reaction by the host. By twenty eight days post-inoculation, all oysters sampled showed an exceptional infiltration of haemocytes. Brown cells were also noted throughout the connective tissue, blood sinuses and outer epithelia. According to Malek and Cheng (16), brown cells appear in case of infection, and play a role in the removal of degradation products of dead or moribund parasites.

Using histological or monoclonal-antibody-based methods, the examination of experimentally infected oyster tissues has not led to the elucidation of the early infective stages of *Bonamia*. Further studies are required to investigate the early stages of infection, possibly by employing electron microscopy or more sophisticated techniques such as *in situ* hybridization.

PART III

MOLECULAR AND CELLULAR STUDY OF STRESS EFFECT ON PARASITE DEVELOPMENT

L. E. Hawkins & S.Hutchinson

CONTENTS

CHAPTER 1

INTRODUCTION

The protozoan *Bonamia ostreae* Pichot has, since the late 1970's and early 1980's, destroyed the commercial exploitation of the European flat oyster *Ostrea edulis* L.in France, northern Spain and most of the grounds in the Netherlands. It is believed that environmental stress is a pre-disposing factor in the occurrence of this disease. The ability to recognise stress is of fundamental importance in the study of any biological system. In seeking to understand the eco-physiology of any species it is necessary to examine the range of functional processes that define the limits of the adaptability of individuals. The use of experimental procedures that measure the effects of environmental changes lying within the normal range of a population gives some indication of the physiological and biochemical basis for the observed distribution of a species and its tolerance of naturally occurring changes; this may be broadly described as a quantitative approximation of a species or population's ecological "fitness". At the level of the individual stress indices give expression to an animal's well-being or "health" (17).

Stress indices have a number of attributes which make them particularly useful in assessing the various responses of marine animals to changing conditions:

1)They represent an integration of the innumerable cellular processes that can alter in response to specific external changes.
2)They can also represent non-specific (generalised) responses to the sum of environmental stimuli that complement more specific cellular or biochemical alterations.
3)They are capable of detecting environmental deterioration from individuals'responses before there are overt effects on a population or community.
4)The rapidity of the response of each index will depend on the rate(s) of the physiological or biochemical process(es) that are measured; and so, each index represents the summation of stresses over the period that is required to cause a measurable change.

Thus, indices can be chosen to show the accumulated effects of a stressor or stressors over periods ranging from many months to a few minutes.

This investigation has incorporated and extended the stress assessment methods and rationale set out by Bayne *et al.*, (17) to establish the effects of various forms of environmental stress on *O. edulis*. The three environmental parameters to be investigated were water temperature, salinity and tidal exposure. Particular emphasis has been laid on the detailed examination of these effects which may reveal stress induced changes in the animal's physiology, biochemistry and immune systems that render them susceptible to pathogens.

This work has also adopted a pluralist approach to indexation since biological systems within animals do not operate in isolation and so no single index can truly reflect the interactions of all the components of an individual's vital processes.

Therefore, experimental procedures have been devised to integrate a wide range of assay methods so that physiological, biochemical and immunological measurements can be made simultaneously on the same animal. This approach not only represents a departure from previous biological indexation methods by integrating them into a single procedure but it also makes use of the functioning of the immune system of *O. edulis*, as part of the indexation methods that were used to measure stress induced by natural environmental variables. The assessment methods used were `Scope for Growth' measurements, biochemical indices and haemocytic indices.

CHAPTER 2

MATERIALS AND METHODS

2.1 MAINTENANCE

Experimental animals of approximately 20-40g wet shell weight were obtained from the Solent, on the south coast of England. Experiments were performed on animals of similar length and weight so as to minimise size-related effects since there is an allometric relationship between size, filtration and respiration rates in *O. edulis* (18,19; Hutchinson, unpublished observation). Prior to experimentation animals were kept in aerated flow-through tanks at ambient water temperature (annual range 12°-19°C) and salinity (33-34 ppt) and fed a mixed algal ration of *Isochrysis galbana, Tetraselmis suecica and Phaeodactylum tricornutum*. This regime was also applied to animals in acclimation and experimental tanks. Animals were acclimated and maintained in microprocessor-controlled, flow-through temperature-salinity systems which allowed continuous and gradual change to the desired salinity/temperature combinations at a rate of 1 ppt per day or 1°C per day away from the stock ambient, whichever was slower. Animals in tidal exposure experiments were maintained in the appropriate experimental conditions for 60 days prior to indexation.

2.2 SCOPE FOR GROWTH

Scope for growth is an overall stress index derived from the energy budget proposed by Winberg (20):

$$C - F = A = R + U + P \qquad (1)$$

or

$$P = A - (R + U) \qquad (2)$$

where:

- C = energy in food consumed
- F = energy lost in faeces and pseudofaeces
- A = assimilated energy
- R = energy lost in respiration
- U = energy lost in nitrogenous excretion
- P = energy available for incorporation into somatic growth and gametogenesis i.e. 'Scope for Growth'

The re-arrangement of the basic equation (*1*) into the second form allows P, the 'Scope for Growth' (21), to be calculated from components on the right-hand side of the equation which are physiological processes that are directly measurable and can be converted to energy equivalents (Joules hour-1).

Animals in favourable conditions will have positive Scope for Growth whereas those animals in stressful conditions will have reduced or negative values as metabolic regulatory requirements exceed the input of energy from assimilation (which also may be impaired by adverse conditions). Chronic exposure to stress will cause the depletion of energy reserves as these are mobilised to cover the difference between energy input and consumption.

The components of the Scope for Growth index were measured using modifications of the procedures given by Bayne (17).

2.2.1 Respiration Rate

Respiration rate was measured in flow-through respirometers supplied with water of appropriate temperature and salinity from the microprocessor controlled system. To measure the respiration rate of each animal water flow was shut off and Schott-Geräte™ oxygen meters and probes were used to measure the linear decrease in oxygen concentration in each chamber. The water in each respirometer was stirred with a magnetic stirrer and maintained at a constant temperature. Values were corrected against a control respirometer and calculated from the equation:

Oxygen consumption rate:

$$(V_{O2}\ \text{mg}\ O_2\ h^{-1}) = (Ct_0 - Ct_1)\ x\ (V_c - V_a)\ x\ \frac{60}{(t_1 - t_0)}$$

V_c = volume of chamber (850 cm^3)
V_a = volume of animal (measured by displacement of water)
t_1 and t_0 are in minutes
Ct_0 = 02 mg l^{-1} at t_1
Ct_1 = 02 mg l^{-1} at t_2

2.2.2 Excretion Rate

The production of ammonia was measured over a 2.5 h period in 250 cm^3 closed vessels by the method of Solorzano (22) corrected against an ambient water control. To determine ammonia concentration 10cm^3 water samples were removed and the following reagents were added to each sample:

-(1) 0.5 cm^3 of 10 g phenol dissolved in 100 cm^3 of 95% ethanol
-(2) 0.5 cm^3 of sodium nitroprusside solution (5 g l^{-1})
-(3) 0.5 cm^3 of oxidizing solution - 25 cm^3 of commercial sodium hypochlorite solution + 100 cm^3 of 20 g trisodium citrate and 1 g of sodium hydroxide dissolved in 100 cm^3 of distilled water.

The contents of each tube were mixed after each addition and the absorbance measured at 640 nm after a 1 hour incubation. The concentration was found from the standard curve given by ammonia standards covering the range 1-10 µM N l^{-1}.

Standards were prepared from a stock standard solution of 1 mM NH_4Cl (= 0.0535 g l^{-1}); 10 µl of stock made up to 10 cm^3 = 1 µM, 50 µl of stock made up to 10 cm^3 = 5 µM and so on.
Results were converted to µg N by multiplying the molar concentration by 14.

Ammonia excretion rate:

$$(\mu g\ NH_4\text{-}N\ h^{-1}) = \frac{(B_a - B_c)\ x\ 14}{1000\ /\ V}\ x\ \frac{1}{t}$$

V = water volume in chamber (250 cm^3)
Bc= NH_4-N in control chamber
Ba= NH_4-N in animal chamber
t = time in hours (2.5h)

2.2.3 Filtration Rate

Filtration rates were measured using a specially constructed upwelling tank in which salinity and temperature were under microprocessor control. It was necessary to acclimate animals to the tank for 2-3 days and reduce disturbance to a minimum so as to avoid inaccuracies caused by animals closing during the course of any measurements. A mixed algal diet was used so as to more closely resemble natural seston, following the observations of Doering & Oviatt, (23) and Bayne *et al* (24); this diet provided a measurable inorganic component that is needed to calculate absorption efficiencies (25). These algal species are greater than 4µm in diameter and therefore of a size at which *O. edulis* can retain 100% of filtered particles (26). It was assumed that there was no preferential uptake of algal species as it is known that there is no size selection ofparticles up to 120 µm in a number of bivalve species (27,28).

The algal ration was set at ~25000 cells cm^{-3} which was maintained by continuous addition with a peristaltic pump. The water flow was maintained at a rate ≥ 250 cm3 per minute for each animal. Algal cells were counted using a Model TAII Coulter Counter.

The filtration rates of individual oysters were calculated from the difference between the number of algal particles in water which had flowed over oysters in the upwelling chambers and the number of particles in the flow from a control chamber.
The clearance rates were calculated from the expression given by Hildreth & Crisp (29):

$$\textit{Clearance rate } (l.h^{-1}) = \frac{C_i - C_0}{C_0} \times F_r$$

F_r = flow rate for each animal $(l.h^{-1})$
C_i = inflow particulate concentration
C_0 = outflow particulate concentration

2.2.4 Absorption Efficiency

Absorption efficiency was determined using methods adapted from those described by Conover (30).
Absorption efficiency (%) (*e*):

$$e = \frac{F-E}{(1-E)F}$$

F = Ash free dry weight : dry weight of food (seston)
E = Ash free dry weight : dry weight of faeces

Physiological rates were then converted into energy equivalents (J h^{-1}) and integrated into Scope for Growth (SFG).

2.2.5 Calculation of Scope for Growth

The following equations describe the calculation procedures used with the different indices.

Scope for Growth (J h^{-1})
$P = A - (R + U)$
$A = C \times e$
C = Clearance rate x POM (mg l^{-1}) x energy of POM*
$R = V_{O2} \times 20.33$

$U = NH_4 \times 0.0249$

*Where POM = Particulate organic matter whose calorific value is taken as 23.5 Jmg^{-1} (31).

The SFG of each individual was then divided by its dry weight in grams sothat data were expressed as $J\ h^{-1}\ g^{-1}$.

2.3 METABOLIC BIOCHEMISTRY ASSAYS

The present study puts into effect the recommendation made by Ivanovici & Wiebe (32) that Adenylic Energy Charge (AEC) should be used in conjunction with other indices of sub-lethal stress; this was done by using additional biochemical indices as well as comparison with Scope for Growth and other independent assays.

AEC was first proposed as an overall stress index by Atkinson (33), who suggested that variations in the ratio of the components of the adenyl-phosphate pool reflected the modulations, by external factors, of enzyme activities at key points in the major metabolic pathways that yield energy in the form of high energy adenine-phosphate bonds. The extension of the biochemical monitoring of stress effects follows the rationale underlying Atkinson's `key enzyme' theory. The concentrations and ratios of a number of metabolites at various points on the Embden-Meyerhof route for glycolysis and Krebs' tricarboxylic acid cycle can be used as indicators of changes in the functioning of the enzymes responsible for the transformations between metabolite pools. This approach gives a range of indices whose integrative periods are related to the hierarchy of energy storage products. The most stable metabolites are glycogen and lipids which are long-term storage products that are present in large concentrations that change slowly in response to seasonal effects; the control of their metabolism in normal conditions is intimately related to the reproductive cycle and they have been used as reproductive condition indices (34). The concentrations of these metabolites change slowly, responding to chronic sub-lethal stress where conditions would be expected to cause a negative Scope for Growth i.e. to draw on energy reserves.

The succinate pool can be considered to be intermediate in the hierarchy of metabolites since, under aerobic conditions, detectable responses occur over a period of hours; tissue concentrations of lactate under aerobic conditions are small in comparison to the other metabolites. However, when *O. edulis* is subjected to anaerobic conditions, caused by valve closure as an avoidance response to adverse environmental conditions, the balance of these metabolites is radically altered and the proportion of lactate is increased whilst the glucose and pyruvate pools are depleted (Unpublished observation). It might be expected that the succinate pool will also decrease during anaerobiosis but, in common with *Crassostrea spp.* and a number of helminth gut parasites, reduced oxygen tension does not completely stop the Krebs'Cycle but halts it at succinate which will accumulate in preference to lactate (35,36,37).

2.3.1 Metabolite Extraction Procedure

Oysters were opened and soft tissues removed, these were carefully blotted to remove excess fluids and wrapped in plastic film ("Clingfilm" or "Saran"). The wrapped samples were immersed in liquid nitrogen. The frozen

tissues were weighed and re-immersed in liquid nitrogen before grinding to a fine powder in a stainless steel (316 grade) mortar and pestle that had been previously cooled in liquid nitrogen. 1 cm^3 of 6% v/v perchloric acid was added to the powdered tissues followed by cooling again in liquid nitrogen. A stainless steel spatula was used to break up the frozen tissue/acid mass and grind it to a powder. The contents of the mortar were transferred to a 50 cm^3 'Nalgene' centrifuge tube kept cold (-20°C) in a freezer. To this was added (weight of frozen tissue x 5) cm^3 of 6% perchloric acid. The mixture was centrifuged for 20 minutes at 4000g, 0-2°C, in capped tubes. After centrifugation the supernatant was decanted into a second set of centrifuge tubes containing 1 drop of bromothymol blue indicator solution; the resulting solution was a pale yellow colour. This was neutralised to pH 7, i.e. until the solution turns pale blue/green, by the dropwise addition of 5M K_2CO_3 solution then centrifuged for a second time at the same settings as before. After the second centrifugation the neutralised supernatant was carefully decanted into cooled graduated tubes and the final volume recorded.

This extract was then used to determine the tissue concentrations of ATP, ADP, AMP, succinic acid and lactic acid and was kept on ice. All analyses were performed immediately after extraction as the extract cannot be stored frozen.

2.3.1 Adenylates and Adenylate Energy Charge (AEC)

Adenylate Tri-, Di- and Monophosphate (ATP, ADP and AMP) tissue concentrations were measured using the procedures described by Bayne *et al.* (17) which are based on the following reactions:

(*1*) ATP + Glucose $\xrightarrow{HK}$ Glucose-6-phosphate (G6P) + ADP

(*2*) G6P + $NADP^+$ $\xrightarrow{G6PDH}$ 6-phosphoglucon-δ-lactone + NADPH + H^+

(*3*) AMP + ADP $\xrightarrow{MK}$ 2 ADP

(*4*) 2 ADP + 2 phosphoenolpyruvate $\xrightarrow{PK}$ 2 ATP + 2 pyruvate

(*5*) 2 pyruvate + 2 NADH + 2 H^+ $\xrightarrow{LDH}$ 2 lactate + 2 NAD^+

G6PDH = glucose-6-phosphate dehydrogenase
HK = hexokinase
LDH = lactate dehydrogenase
MK = myokinase
NAD = ß-nicotinamide-adenine dinucleotide, oxidised form
NADH = ß-nicotinamide-adenine dinucleotide, reduced form
$NADP^+$ = ß-nicotinamide-adenine dinucleotide phosphate, oxidised form
NADPH = ß-nicotinamide-adenine dinucleotide phosphate, reduced form

ATP was measured by following reactions (*1*) and (*2*). ADP and AMP werefound from reactions (*3*)-(*5*). These concentrations were used to calculate the Adenylate Energy Charge (AEC), as defined by Atkinson (33):

$$AEC = \frac{[ATP] + 1/2\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

annexe 1.

ATP was analysed by itself in one set of cuvettes, while ADP and AMP were analysed sequentially in a separate set of cuvettes. The cuvettes with samples and standards were read against a reagent blank for each of the analyses. This corrected for any changes in absorbance that might be caused by various additions of enzymes.

ATP assay

2.85 cm^3 of ATP assay buffer was dispensed into each cuvette. 0.1 cm^3 portions of samples and standard mixture were dispensed in duplicate with one or two blanks. 10 µl of G6PDH was added to each cuvette and stirred well to ensure that there were no air bubbles inside each cuvette. The absorbance (A1) was read after 5- 10 min. This allowed sufficient time for oxidation of any endogenous G6P that might have been in the sample. The addition of 20 µl of glucose was followed by 20 µl of HK. 10-20 min at room temperature were allowed for the reaction to reach completion (i.e. when there were no further changes in absorbance on the recorder), and then A2 was read.

Neutralized Supernatant

ATP Assay	ADP, AMP Assay
0.1 cm^3 into cuvette	0.2 cm^3 into cuvette
+	+
2.85 cm^3 assay buffer	2.8 cm^3 assay buffer
+	+
10 µl G6PDH	5 µl LDH, mix
Read A1	Read B1
20 µl glucose,mix immediately add 20 µl HK,mix	5 µl PK, mix; read B2 when reaction complete
Read A2 when reaction complete	5 µl MK, mix; read B3 when reaction complete

ADP-AMP assay

2.8 cm^3 of ADP-AMP assay buffer was dispensed into each cuvette followed by 0.2 cm^3 of sample, 0.1 cm^3 of standard mixture plus 0.1 cm^3 water, and 0.2 cm^3 of water for the blank. 5 µl LDH was then added to all cuvettes, mixed well to remove bubbles and the absorbance (B1) read after 5-10 min. 5 µl PK was added to each cuvette and B2 was read after the reaction was complete (5 min). Finally, 5 µl MK was added and B3 read after 15-20 min. Since this last reaction was the slowest of the three, a check with the recorder for the reaction's completion was necessary.

Check for Enzyme Activities

New enzymes for both assays were checked for activity before embarking on a series of assays. For enzymes used in the ATP assay a standard mixture used consisting of 0.05 mM G6P and ATP, 0.1 cm^3 of this was added to a cuvette with 2.85 cm^3 of ATP assay buffer. The absorbance (A0) was read before adding G6PDH which was then added and A1 read at the completion of the reaction.

The difference (A1-A0; G6PDH) should be approximately 0.100 if the G6PDH was active. The difference (A2-A1; ATP) should also be 0.100 if the HK was active. For enzymes used in the assay for ADP and AMP a mixture was made of 0.05 mM pyruvate, ADP and AMP. B0 was read before adding LDH. The difference (B0-B1; pyruvate) should be approximately 0.100 if LDH was active for a volume of 0.1 cm^3 of standard mixture. Similarly (B1-B2; ADP) and (B2-B3; AMP) should be approximately 0.080-0.100 and 0.200, respectively.

The calculation of nucleotide concentrations is presented in annexe 1 bis.

2.3.2 Succinate

Succinate measurements were based on the method given by Beutler (38):

$$\text{Succinate} + \text{CoA} + \text{ITP} \xrightleftharpoons{\text{SK}} \text{succinyl-CoA} + \text{IDP} + P_i$$

$$\text{IDP} + \text{PEP} \xrightarrow{\text{PK}} \text{ITP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+$$

CoA = coenzyme A
ITP = inosine-5'-triphosphate
IDP = inosine-5'-diphosphate
PEP = phosphoenolpyruvate
SK = succinic thiokinase

The different solutions used (for 20 assays) are presented in annexe 2

Assay

A reagent blank with water instead of sample is essential because of a small content of "NADH oxidases" in succinyl-CoA synthetase and because of a slight decomposition of ITP to form IDP (consumption of NADH). For the measurements, each cuvette contained:

- glycylglycine buffer (1.00 cm^3)(1)
- NADH solution (0.10 cm^3)(2)(2)
- coenzyme solution (0.10 cm^3)(3)
- enzyme mixture (0.05 cm^3)(4)
- sample (0.10 cm^3)
- water (1.50 cm^3)

The contents of each cuvette were mixed thoroughly with a plastic spatula and the change in absorbance monitored until constant (5-20 min) and then the absorbance A1 was read. Then was added:

- succinyl-CoA synthetase (0.02 cm^3)(5)

After completion of the reaction (ca. 30 min), absorbance A_2 of the solutions was read immediately one after the other. Completion of the reaction is reached when sample and blank show equal changes in absorbances.

Calculation: to calculate the absorbance differences (A_1 - A_2). Use $\Delta A = (A_1 - A_2)_{sample}$.

$$Succ.conc.g\text{-}1\ wet\ weight = \frac{(vol.\ of\ total\ extract\ (cm^3) \times \Delta A) \times 1.9855}{Wet\ weight\ of\ soft\ tissue}$$

2.3.3 Lactate

Lactate was measured by method given by Noll (39) using the coupled reactions:

$$\text{L-(+)-lactate} + NAD^+ \xrightleftharpoons{LDH} \text{pyruvate} + NAD + H^+$$

$$\text{Pyruvate} + \text{L-glutamate} \xrightleftharpoons{ALT} \text{L-alanine} + \text{2-oxoglutarate}$$

ALT = L-alanine:2-oxoglutarate amino transferase

The preparation of solutions (for 40 assays) are presented in annexe 3.

Assay:

Wavelength 340 nm; light path 10 mm; final volume 3.0 cm^3; temperature 25°C in a constant-temperature cell holder; measurement against water and a blank* with $(NH_4)2SO_4$ solution, 1.8 M, instead of ALT suspension and with water instead of sample.

* Since even in the absence of L-(+)-lactate a slight increase in absorbance of 0.005-0.010 occurs, it is finished 5 min after addition of LDH suspension (6). This blank has to be measured. Its value depends on the concentration of glutamate, but is independent of the concentration of NAD and of the presence of ALT.

Measurement

Each cuvette contained:

- glutamate buffer solution (0.67 cm^3)(2)
- NAD solution (0.10 cm^3)(4)
- sample (deproteinized, neutralized) (0.20 cm^3)
- ALT suspension (0.05 cm^3)(7)
- water (1.96 cm^3)

The contents of each cuvette was mixed thoroughly with a plastic spatula, incubated for 10 min at 25°C and absorbance A_1 read. Then was added:

- LDH suspension (0.02 cm^3)(6)

Absorbance followed until the constant final absorbance A_2 is reached (usually after 15-30 min) and $A_2-A_1 = \Delta A'$ used for the calculation.

2.4 HAEMOCYTIC AND HAEMOLYMPH INDICES

Quantitative assessments were made of the effects of stress on the haemocytes of *O.edulis* since these are the main cellular defences against pathogens (40); lysozyme and hydrogen peroxide are produced as non-specific microcidal agents and haemocyte motility is a measure of the ability of the oyster to eliminate foreign material by phagocytosis and subsequent diapedesis. The integration of lysozyme, hydrogen peroxide and haemocyte

motility measurements into the assays of metabolic stress effects provided direct observations of the exact relationship between the functioning of oyster defence mechanisms and the rest of the animal's life processes. In this way it was possible to bring together a number of previously disparate observations by incorporating them into a multi-system model of oyster responses to environmental stressors. For example, bivalve haemocytes can remain active during periods of anoxia caused by valve closure (41) but also succinic acid is accumulated in preference to lactic acid under the same conditions (35). The accumulated succinic acid can be used by the haemocytes to sustain their activities whilst the rest of the tissues are quiescent but prolonged anoxia or high temperatures cause greater lactic acid production and a consequent reduction in haemocyte activity.

The integration of metabolic indexation with the quantitative study of haemocyte-mediated defences made it possible to explain differences in disease susceptibility and other physiological responses of *O. edulis* in terms of the effects of stressors on the metabolic processes controlling haemocyte functions.

2.4.1 Sampling Procedure

Samples of haemolymph were taken from the adductor muscle sinus by aspiration into a drawn-out and acid-cleaned Pasteur pipette and placed in a covered embryo dish previously coated with silicones using Repelcote™.

In vitro measurements of live haemocytes were made using a calibrated video microscopy system in conjunction with Nomarski differential contrast interference optics to visualise the cells. 50 µl of haemolymph was diluted with an equal volume of filtered seawater at the acclimation temperature and placed on a 'Neubauer' haemocytometer. The dilution with seawater facilitates cell counting and appears to promote the initial aggregation of haemocytes without the introduction of potential artefacts caused by the manipulation of calcium and other divalent cations in the diluent to control cell movement, as used in similar methods described by Fisher & Newell (42). Haemocytes can be identified and classified into three types according to a scheme adapted from that proposed by Auffret (43) for *Ostrea edulis* and equivalent to that used by Chagot (44):

Hyalinocytes - small, dense, agranular and spherical cells 2-4µm diameter, producing hydrogen peroxide on contact with foreign particles by 'bursts' of respiratory activity.

Granulocytes - spherical cells containing numerous dense granules and 8-10 µm diameter.

Amoebocytes - irregularly shaped, motile cells often containing a small number of granules and when aggregated 10-15 µm in diameter but capable of producing pseudopodia 2-3 times this size and engulfing foreign material.

Video recordings of the dispersal of haemocyte aggregations permit the subsequent measurement of cell dimensions, the rates of locomotion of individual cells following the dispersion of aggregates as well as counts of the three cell types.

The remaining portion of the haemolymph sample can be assayed for hydrogen peroxide concentration and lysozyme content.

2.4.2 Hydrogen Peroxide Concentration

This is done spectrophotometrically, using the method described by Meiattini (45). It is possible to determine the peroxide concentration of a given sample by using a suitable hydrogen donor which is colourless when reduced, but turns blue on oxidation. The reaction is given by :

$$H_2O_2 + DH_2 \qquad 2H_2O + D$$

For this determination the hydrogen donor (DH_2) is 4-amino-phenazone/chromotropic acid. The intensity of colour produced is proportional to the concentration of peroxide in the solution. The absorption coefficient varies with the experimental conditions so the absorbance of the sample is compared with the absorbance of a standard solution.

Haemolymph samples were deproteinized within 1-2 minutes, because other proteins in the sample would affect the assay (hydrogen peroxide is unstable in some protein-containing solutions). To deproteinize the sample, 0.10 cm^3 of haemolymph was pipetted into a centrifuge tube, together with 0.10 cm^3 of 6% perchloric acid. This was centrifuged at 2000 g for 5 minutes. After this, the supernatant was decanted off into a test tube, where the acid was neutralised by addition of 100 µl of 6 M sodium hydroxide solution. 100 µl of this solution was then used in the assay. The preparation of the different solutions is presented in annexe 4.

2.5 cm^3 of peroxide reagent was placed in each cuvette, together with 0.1 cm^3 of either distilled water (the reagent blank), H_2O_2 standard solution, or deproteinized sample. After mixing the mixture was allowed to stand for 5 minutes at room temperature. The absorbance of the sample or standard was read against the reagent blank within 20 minutes.

The absorbance of the samples was read at a wavelength of 600 nm in 3 cm^3 cuvettes with a light path of 10 mm.

The hydrogen peroxide concentration of the haemolymph samples was found by comparing the absorbance of the sample with that of the standard H_2O_2 solution (20 mg l^{-1}), multiplied by the dilution factor - for the deproteinized samples, this factor was 2.1.

$$\textit{Mass Concentration } (\rho) = \frac{sample}{standard} \; x \; 20 \; x \; 2.1 \; mg \; l^{-1}$$

2.4.3 Lysozyme Concentration

This is also done spectrophotometrically, using the method described by Weisner (46). This assay is based on the ability of lysozyme to lyse freeze-dried *Micrococcus luteus* in suspension at a known concentration. This reduces the turbidity of the sample which can be measured photometrically. The extent of reduction of turbidity is proportional to the catalytic activity of lysozyme in the assay mixture.
The detailed protocol is described in annexe 5

Fresh haemolymph samples were centrifuged immediately at 2000 g for 10 minutes, and then analysed within a few minutes. The absorbance was

measured at a wavelength of 546 nm, using 3 cm^3 cuvettes, with a light path of 10 mm. 0.025 cm^3 of haemolymph sample was added to each cuvette with 1.5 cm3 of the *Micrococcus* suspension and timing started. Each sample was thoroughly mixed with a glass rod. The absorbance was read after exactly 30 seconds (A_1), 120 s (A_2), and 210 s (A_3).

To calculate the lysozyme activity for each sample, the change in absorbance with change in time was calculated. This is given by :

$$\frac{\Delta A}{\Delta t}\ (90\ s)^{-1} = \frac{[(A1 - A2) + (A2 - A3)] / 2}{\Delta t}$$

-where Δt = 90 seconds.

The catalytic activity of lysozyme in each sample is given by:

$$x = \frac{y - a}{b}\ E.\ U.$$

-where y = $\Delta A/\Delta t$, and a and b are constants, derived from the linear regression of the activities of the standards.

-E.U. = Enzyme Units. One unit is defined as that amount of enzyme which causes a $\Delta A/\Delta t$ of 0.001 per minute (450 nm; light path 10 mm; potassium phosphate 0.1M, pH 7.0; 25°C; substrate as above).

2.4.4 *In vitro* Examination of the Effects of Elevated Lactate Concentration on Haemocyte Activity

A 1 µM solution of lithium lactate in filtered sea water was made by serial dilution. Lithium lactate was used as the use of the sodium salt would increase the sodium concentration of the sea water, and the calcium salt has a known inhibitory effect on the movement of cells by disrupting microtubule formation. A final incubation concentration of 0.09 µmol g-1 wet tissue weight of lactate was used to reproduce the lactate concentrations found in *O. edulis* exposed to the air for 3 hours at 15°C.

Samples of haemolymph (approximately 300 µl each) were taken from oysters kept fully submerged in well aerated water so as to provide haemolymph with zero or minimal lactate concentrations. The soft tissues of each oyster were removed, blotted to remove excess water, and then weighed. The soft tissue weights were used to calculate the amount of 1 µM lactate/sea water solution that is required for dilution to give an equivalent concentration of 0.09 µmol g^{-1} when 50 µl of haemolymph was mixed with 50 µl of lactate/sea water.

A control was provided by mixing 50 µl of haemolymph with an equal volume of filtered sea water without lactate. The pH's of the mantle fluid and measured as should the sea water containing lactate.

CHAPTER 3

THE EFFECTS OF TEMPERATURE ACCLIMATION

The results have been grouped according to the major stressor and sub-divided within each group into:

1)Physiological measurements of whole animals i.e. Scope for Growth indexation.

2)Quantification of metabolic state derived from metabolite and adenylate phosphate concentrations.

3)Measurements of haemocytic activity.

Measurements were made on oysters acclimated to 5, 15 and 25°C and 34K or 16, 25 and 34K at 15°C. The number of animals at each datum point (n) = 36. Animals were maintained in the flow-through conditions at the required temperature salinity and fed a standard algal ration described in the Chapter 2. Data points represent mean values, error bars indicate standard error (S.E.).

3.1 SCOPE FOR GROWTH MEASUREMENTS (Figures 18-22)

The physiological responses of *Ostrea edulis* to the three acclimation temperatures are shown in Figures 18 to 22, and on the basis of these results, 15°C would seem to be the most favourable (least stressful) temperature since it is associated with the maximum mean scope for growth value of 142.56 ± 6.48 J h^{-1} g^{-1} dry wt.

The energy available to a bivalve under a given set of conditions will depend on absorption efficiency, filtering rate and seston concentration; the absorption efficiency of *O. edulis* showed little change with respect to temperature. A similar result was obtained by Widdows & Bayne (47), who found that *Mytilus edulis* showed no change in absorption efficiency following 14 days acclimation to high and low temperatures and by Buxton *et al.* (48), using *O. edulis* acclimated to temperatures between 5°C and 20°C. The results presented here are further confirmation of the ability of many poikilotherms to regulate their metabolism by acclimation when under thermal stress (49).

The small scale of the changes in absorption efficiencies (Fig 18) in *O. edulis* indicates that the energy input to the experimental animals was mainly controlled by the responses of filtering capacity to temperature and salinity. Bivalves may regulate food intake in two ways (28); intake can be kept constant with increasing food concentration if the filtering rate is not modified but excess food is rejected in the form of pseudofaeces as suggested by Widdows *et al.*(31), in a study of *M. edulis*. Alternatively, intake can be limited by a reduction in filtering rate in response to increasing food concentrations which is consistent with the results obtained from *O. edulis* by the present study and that of Mathers (50), as well as those of *M. edulis* by Davids (51), Foster-Smith, (28), Winter (52) and Schulte (53). Also, Loosanoff & Engle (54) found that the pumping rates of *C. virginica* were reduced as particle concentration increased. The data that were obtained for filtering rates of *O. edulis* in 34K seawater are similar to those obtained by Loosanoff (55) from *Crassostrea virginica* and J¢rgensen *et al.* (56), working on *Mytilus edulis*. It has been suggested by J¢rgensen (25) that this type of response is mainly attributable to the inverse relationship between temperature and the viscosity of water.

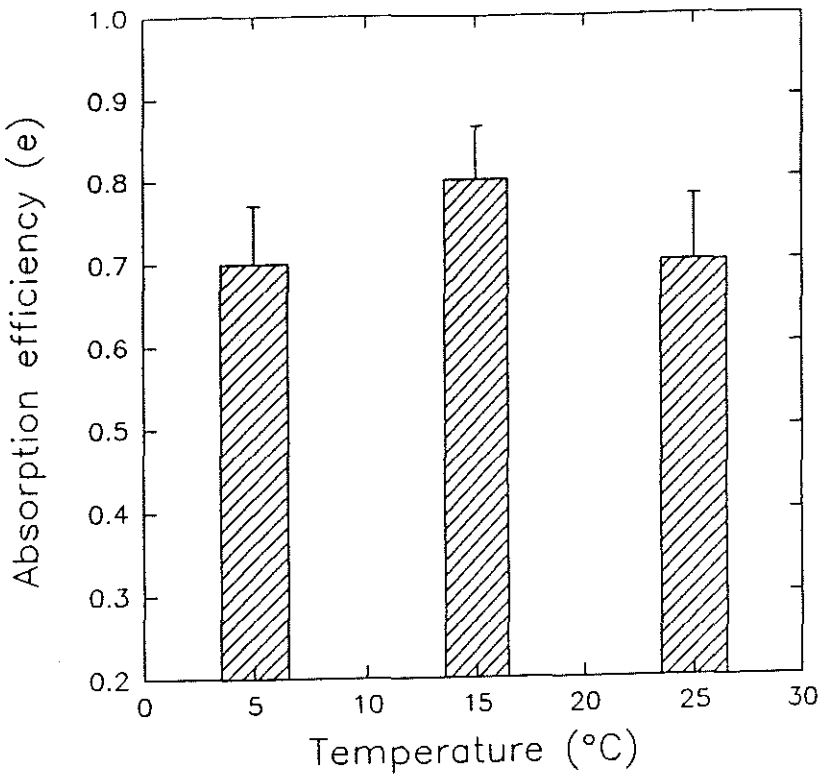


Figure 18. Absorption efficiencies of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

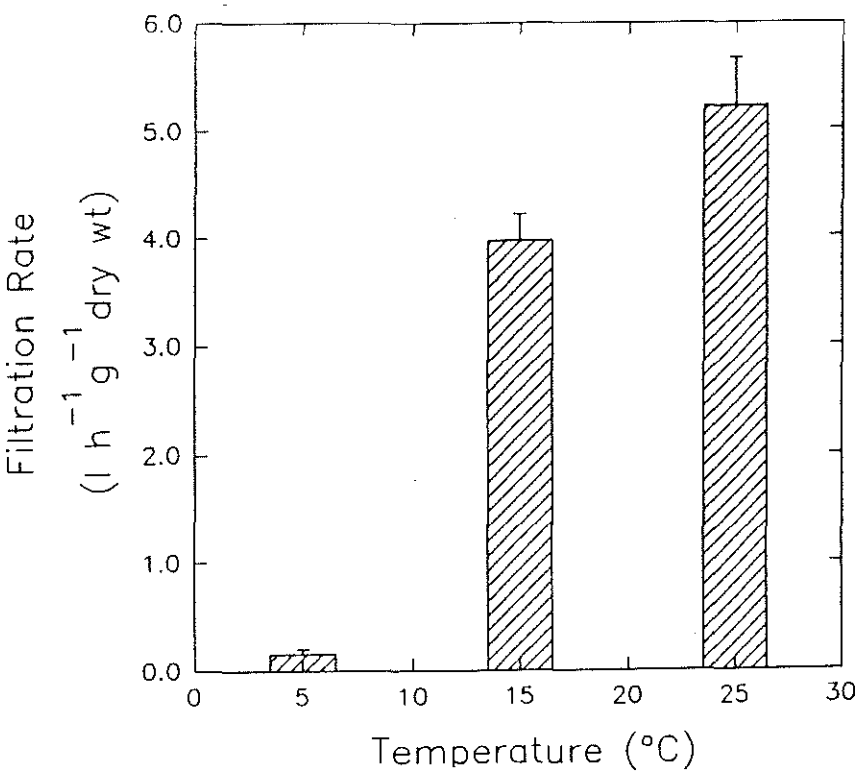


Figure 19. Filtration rate of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

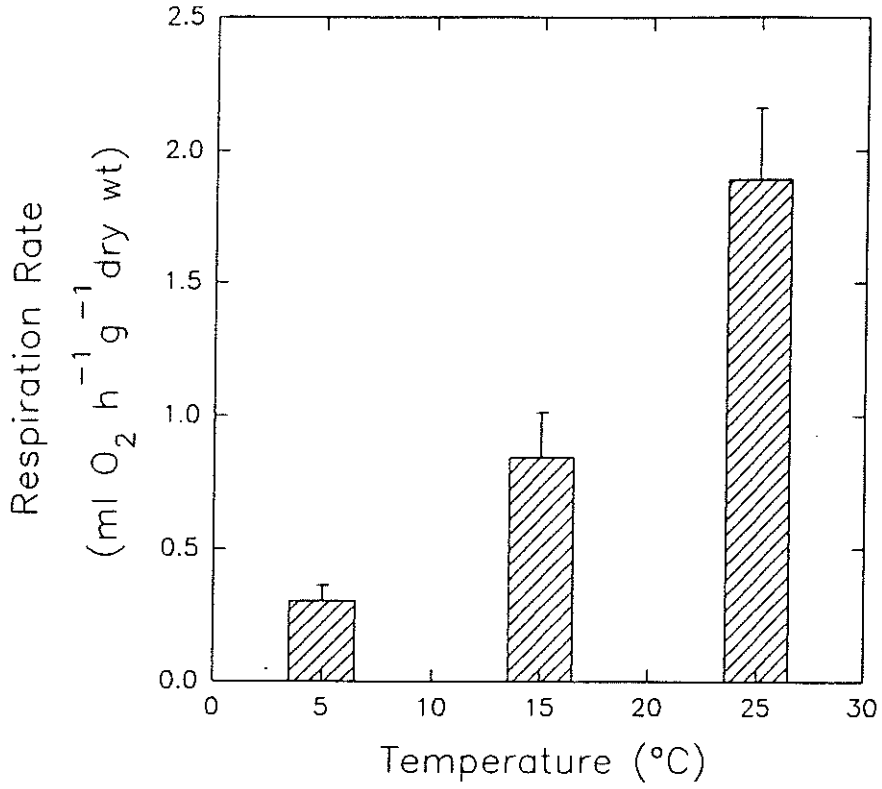


Figure 20. Respiration rate of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

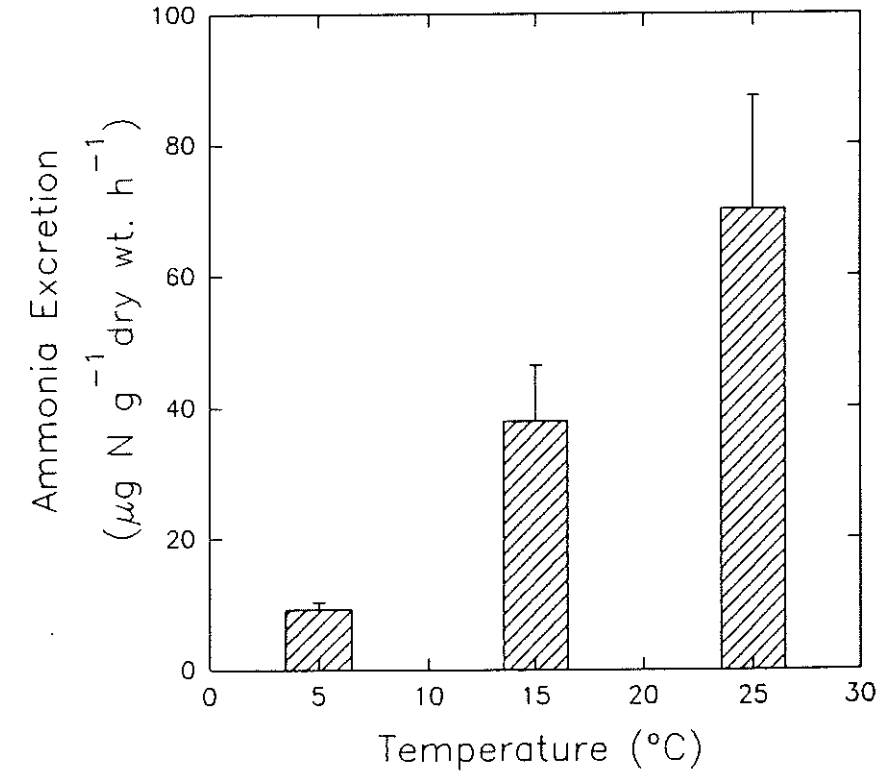


Figure 21. Ammonia excretion rates of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

The energy consumption component of Scope for Growth in *O. edulis* is dominated by the energy expenditure represented by oxygen consumption. Respiration rates have been divided into standard, routine and active oxygen consumption by many authors (57,59,48) but J¢rgensen *et al.* (25,60) attribute the different respiration values to the physico-chemical relationship of passive diffusion with variations in ventilatory activity depending on the presence of particles in the water column. In the work presented here respiration rates were measured in animals deprived of food for 24 h and in the absence of particulate matter. In the temperature acclimation experiments there appeared to be a discontinuity between 5°C and 15°C; this can be ascribed to a switch between 'summer' and 'winter' physiological states at temperatures around 10°C. These states have already been described in *Crassostrea virginica* (61) and they can be characterised by changes in the metabolic biochemistry of *O. edulis* (Hawkins & Hutchinson, in preparation). There was no evidence of thermal acclimation of oxygen consumption rates in *O. edulis* which is concordant with the observations of Newell *et al.* (59) and Buxton *et al.* (48). However, it is difficult to separate somatic responses to water temperature from the processes of gametogenesis since the induction of gametogenesis can be related to changes in water temperature and seasonal changes in oxygen consumption related to gametogenesis in *M. edulis* were reported by Bayne & Thompson (62).

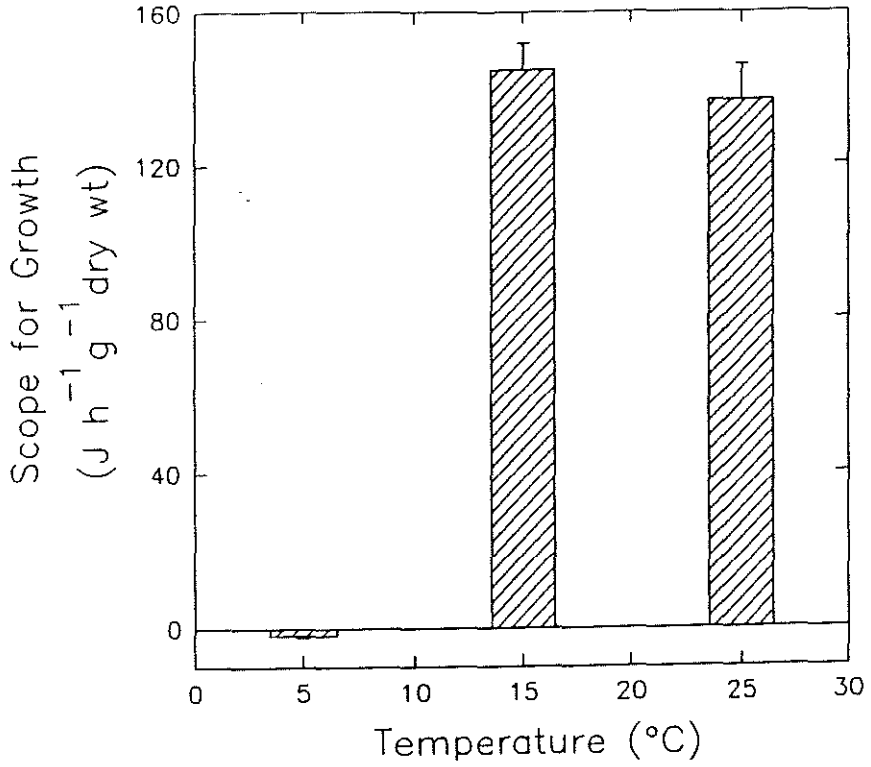


Figure 22. Scope for growth values of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

The nitrogenous excretion term of the SFG equation is small when converted into energy equivalents but the variations in rates of nitrogenous excretion give some indication of the nature of the physiological responses to given levels and combinations of stressors.

From the analyses of the Scope for Growth index and its components it can be seen that filtering rates and oxygen consumption determine the energy input components of scope for growth indices and these are most influenced by water temperature. It is a predominantly physico-chemical relationship between water temperature and filtering rate which is modulated by biological effects at the extremes.

3.2 METABOLIC EFFECTS (Figures 23-26)

The metabolite concentrations are concordant with the scope for growth results, reflecting the shifts in balance between energy loss and gain. This is exemplified by the changes in the concentrations of adenosine triphosphate (ATP) and adenosine diphosphate (ADP). These are at a maximum at 15°C and adenosine monophosphate (AMP) concentration was at a minimum which results in a high Adenylic Energy Charge (AEC) at this temperature. This suggests that rephosphorylation activity was maximal at this temperature and salinity.

3.3 HAEMOCYTIC EFFECTS (Figures 27-34)

N.B. The term "amoebocyte" is used here to define large, motile granulocytes.

These results indicate that 15°C would seem to be a optimal temperature according to the above apparent measures of cell-mediated immunity. It can also be seen that there are relationships between variations in the amoebocyte and hyalinocyte populations and the effects of acclimation temperature on the titres of haemolymph lysozyme and hydrogen peroxide. The former seems to vary with amoebocyte number and the latter seems to be related to hyalinocyte numbers, implying a segregation in the secretory activities of these two cell populations.

The mean rates of amoebocyte locomotion can be considered to be indicators of the metabolic activity of this population of haemocytes. The metabolic activity of these cells will affect their potential phagocytic activity and so may be considered to be an index of immunocompetence. Although the rates of locomotion increased with temperature these increments were small and non-significant ($P > 0.05$). Given that there is evidence of switching between metabolic states in the somatic tissues it would seem that amoebocytes are relatively unaffected by temperature changes, this can be ascribed to a difference in the thermal sensitivities of the key enzymes of somatic and haemal cells. Dramatic differences have previously been shown in the metabolism of somatic and haemal cells of the North American oyster *Crassostrea virginica* (41). The relative thermal insensitivity of the amoebocytes has considerable ecophysiological implications since it would suggest that phagocytic activity could be maintained at low water temperature i.e. during winter months. It remains to be determined whether other types of haemocyte also show this thermal acclimation.

Variations in lysozyme production are consistent with the concept of low temperature acclimation of amoebocytes since the haemolymph titres at 5°C and 15°C show only a minor difference. However, at 25°C there is a reduction in lysozyme activity which could either be the result of a

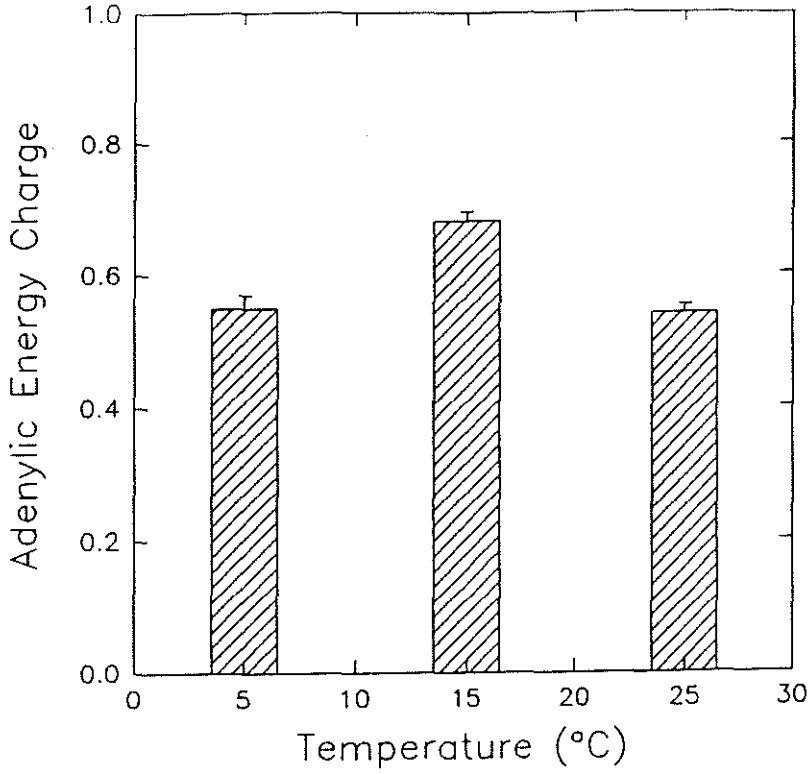


Figure 23. Adenylic Energy Charge of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

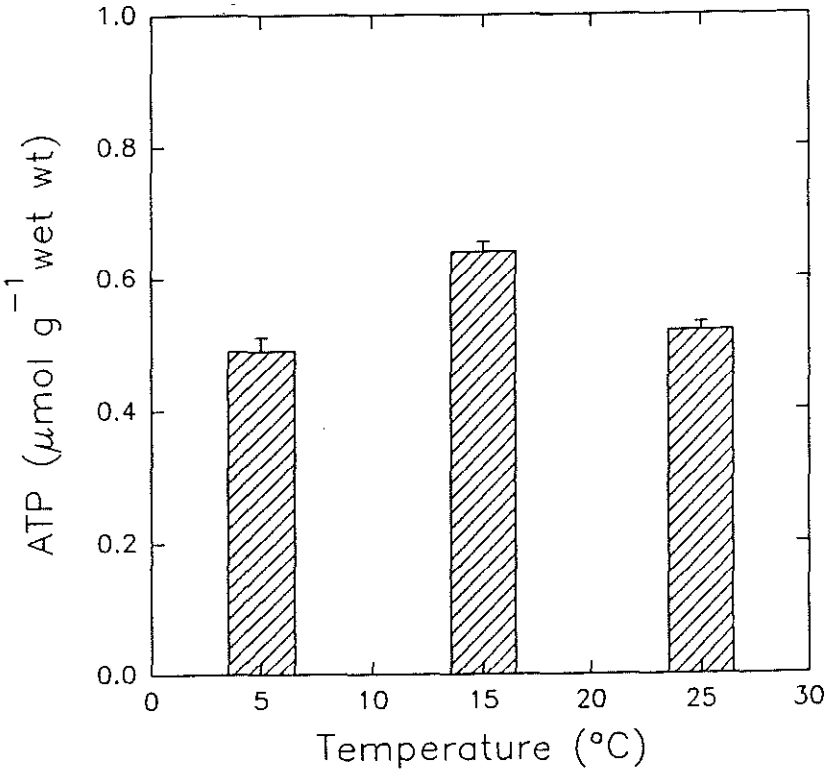


Figure 24. Adenosine triphosphate concentrations in the tissues of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

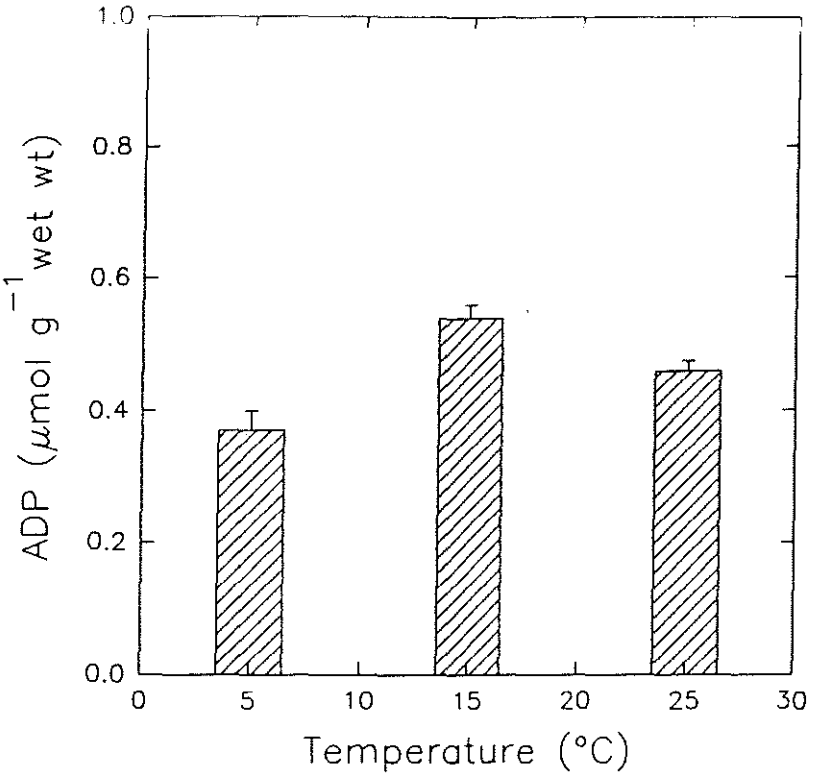


Figure 25. Adenosine diphosphate concentrations in the tissues of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

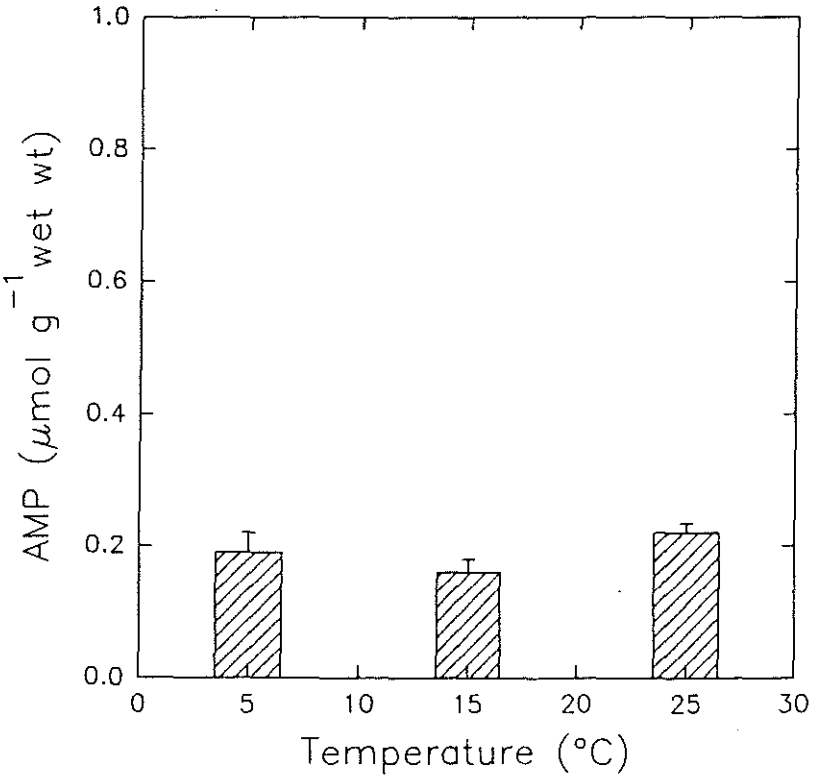


Figure 26. Adenosine monophosphate concentrations in the tissues of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

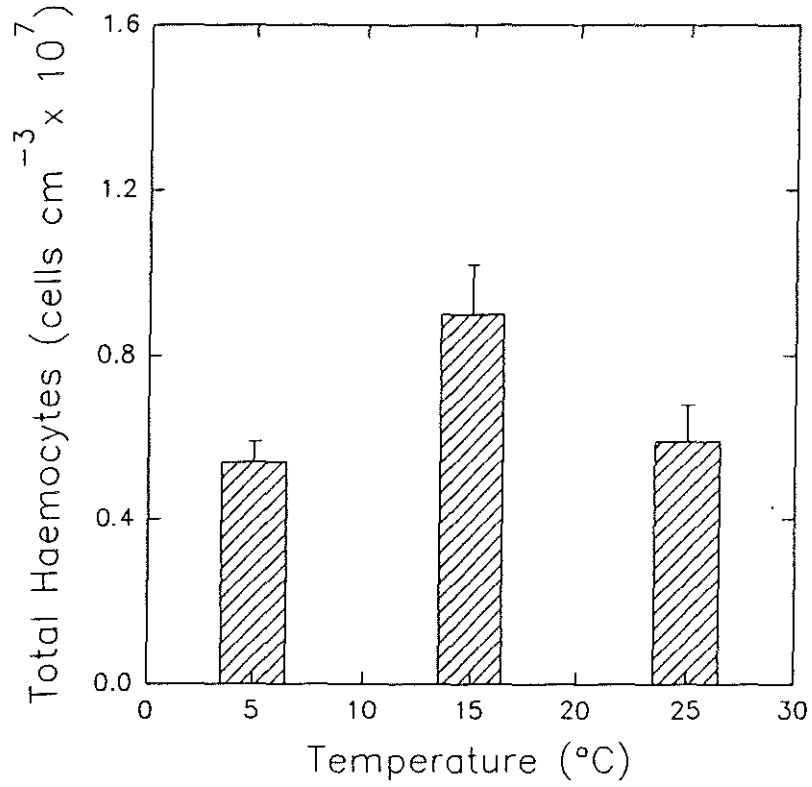


Figure 27. Total haemocyte concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

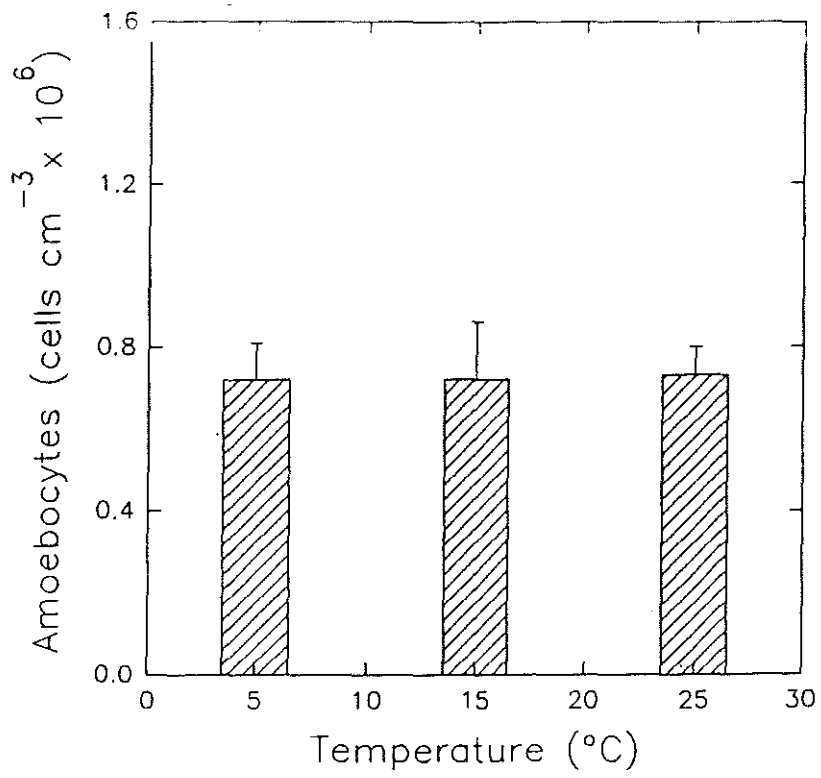


Figure 28. Total amoebocyte concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

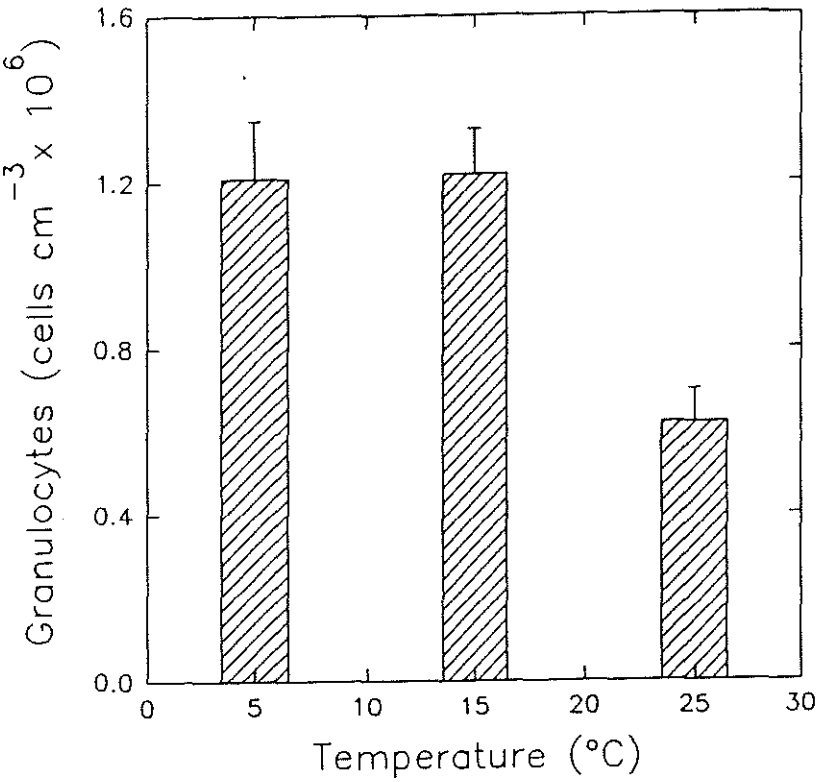


Figure 29. Total granulocyte concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

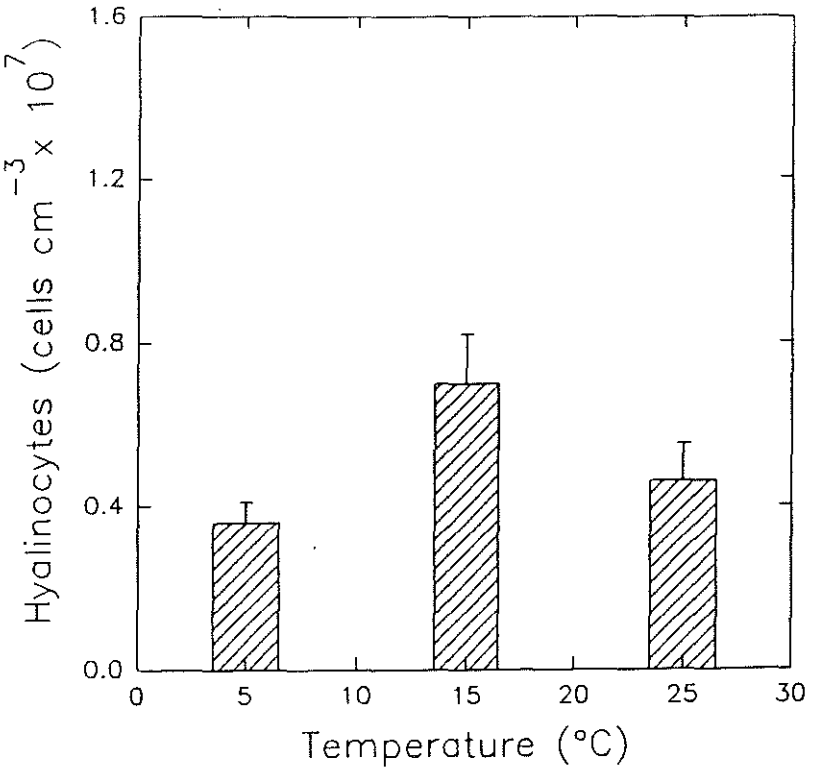


Figure 30. Total hyalinocyte concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

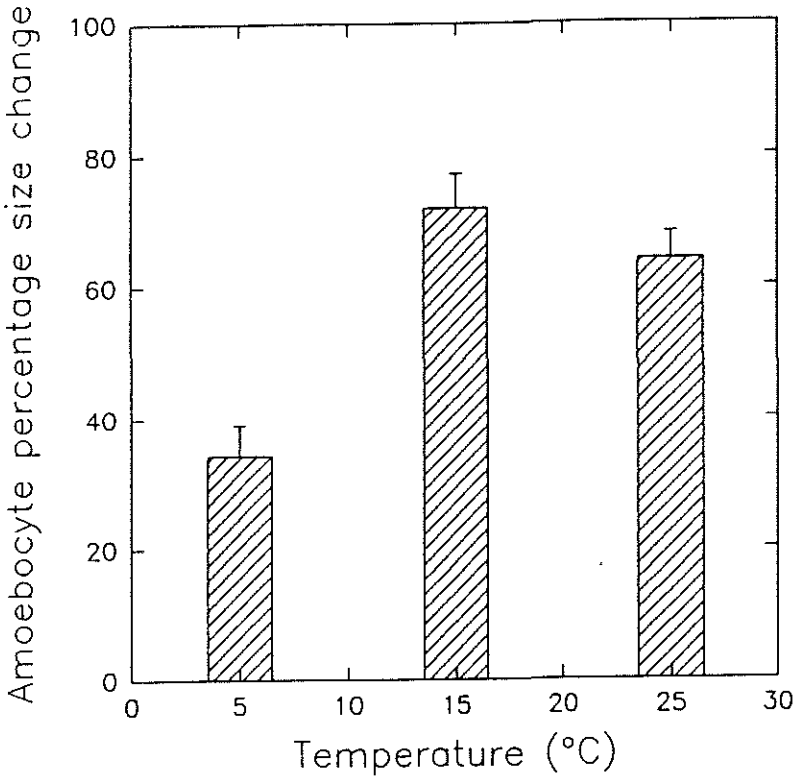


Figure 31. Amoebocyte percentage cell size changes of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

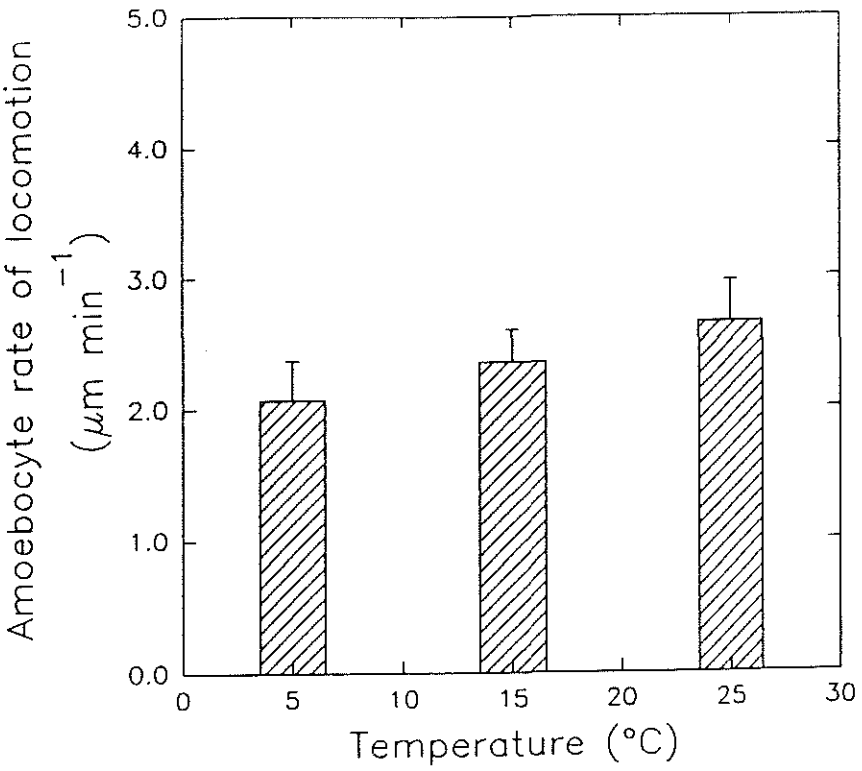


Figure 32. Amoebocyte rates of locomotion of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

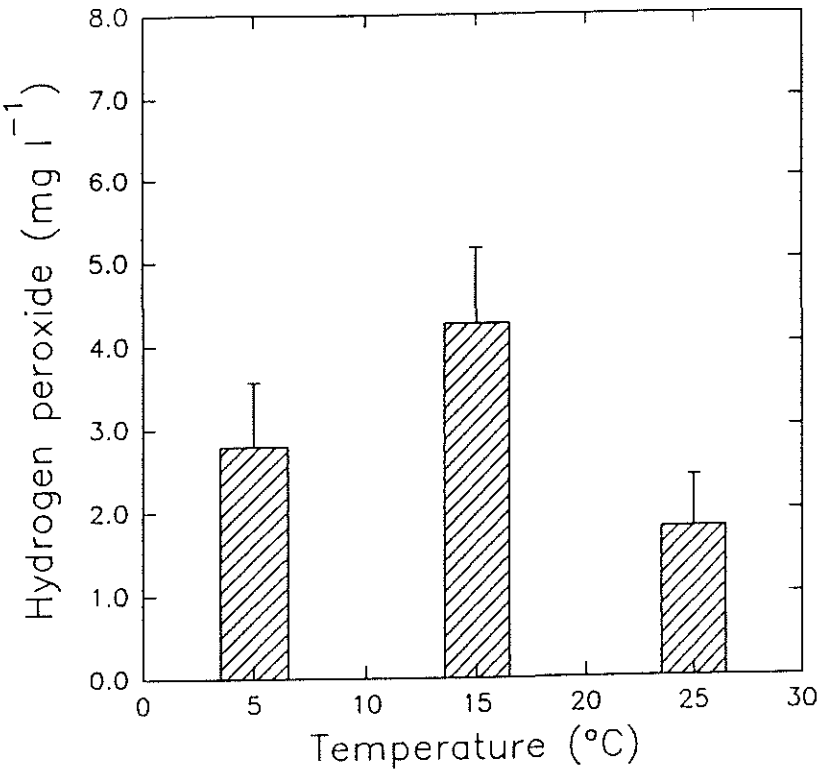


Figure 33. Haemolymph hydrogen peroxide concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

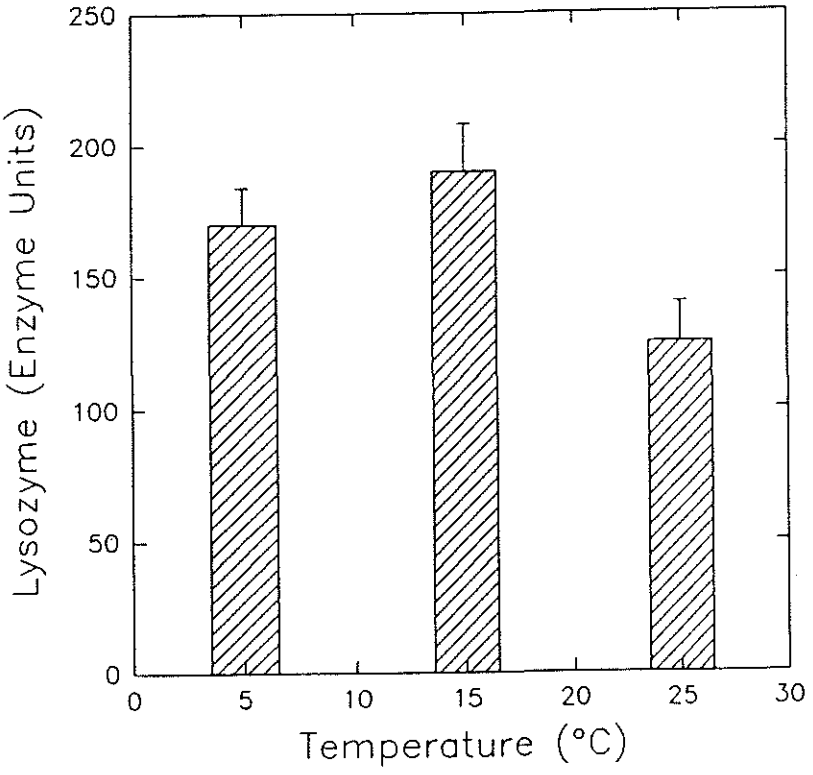


Figure 34. Haemolymph lysozyme concentrations of *Ostrea edulis* acclimated to 5, 15 and 25 °C and fixed salinity (34 ‰).

reduction in lysozyme synthesis caused by the reduction in the scope for growth at 25°C or by intrinsic changes in enzymatic activity caused by temperature-induced changes in the conformation of lysozyme proteins.

CHAPTER 4

THE EFFECTS OF SALINITY ACCLIMATION

4.1 PHYSIOLOGICAL MEASUREMENTS (Figures 35-39)

The results shown in Figure 18 show that temperature exerts little effect on absorbtion efficiency but it is reduced at the lower end of the salinity range that was used (Fig 35). This may be attributable to the fact that as an osmo-conformer (63), *O. edulis* lacks the capability of isolating its non-regulatory metabolic processes from effects of exposure to changes in salinity.

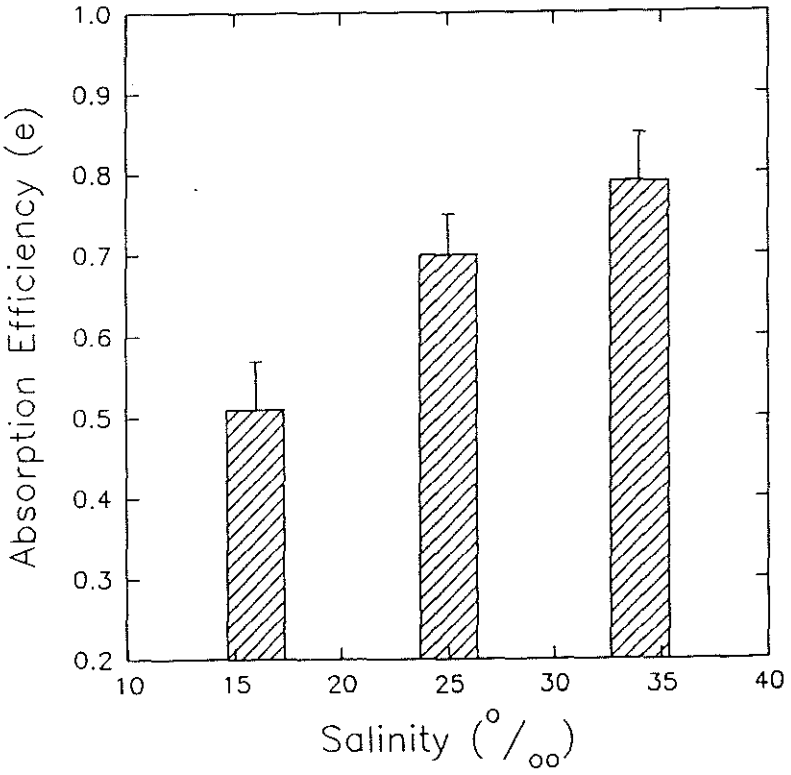


Figure 35. Absorption efficiencies of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

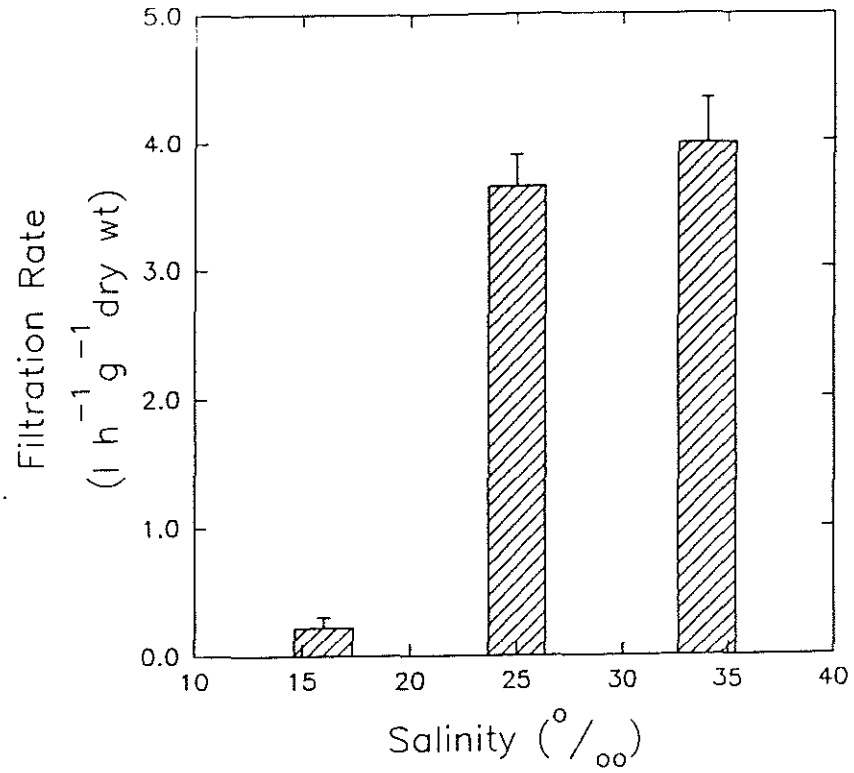


Figure 36. Filtering rates of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

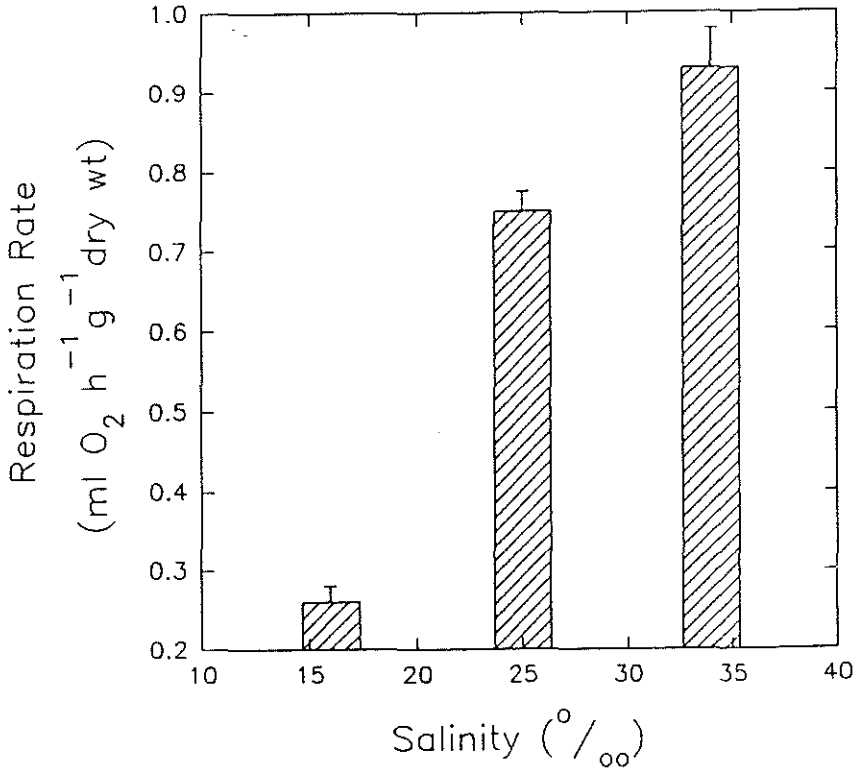


Figure 37. Respiration rates of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

It has been suggested by Jœrgensen (25) that variations in the filtration rates of bivalves mainly reflect viscosity changes in the inhalant water but the results obtained from animals at reduced salinities show departures from this purely physico-chemical relationship and it should be noted that at temperatures ≥ 15°C salinity has only a minor effect on the density of seawater (64). It may be concluded from this that *O. edulis* substantially modifies its ability to filter at reduced salinities; it is presumed that these reductions are the consequence of periods of valve closure by which means bivalves are able to mitigate the effects of exposure to reduced salinities (65; 66, 67), although reduced irrigation of the gills would have the same effect.

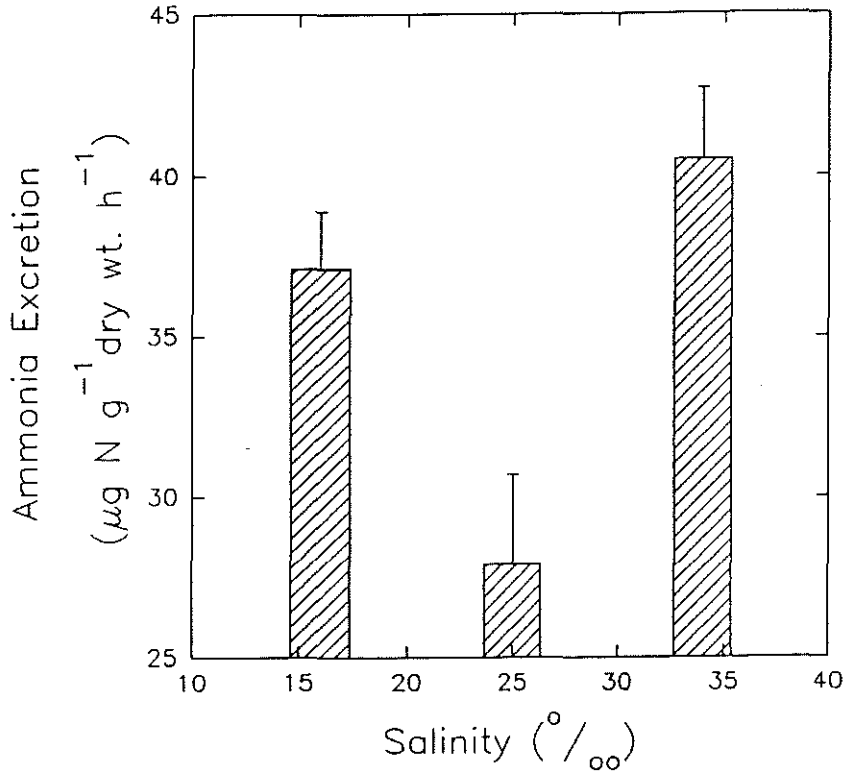


Figure 38. Ammonia excretion rates of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

Reduced salinity was found to be associated with decreased oxygen consumption. This is contrary to the response of *M. edulis* to decreasing salinity which shows a linear increase in oxygen consumption between 30 ppt and 10 ppt (68). The reduction in oxygen consumption in *O. edulis* may be caused by increased duration of valve closure in response to reduced salinity, stopping both the feeding current and ventilatory gill irrigation. It must be emphasised that the findings of this study were based on observations of animals acclimated to reduced salinities since the

stabilized metabolic rate of well acclimated animals may be completely different to the temporary response to direct salinity transfer or short term salinity fluctuations (69;70).

The variations in ammonia excretion in response to reduced salinity may be taken as an indication of increased metabolic turnover of nitrogenous compounds related to osmoregulation by mobilisation of intracellular free amino acids (71,72,73,74,75 and 76).

The scope for growth measurements suggest that *O. edulis* is able to accommodate reduced salinities between 25 ppt and 34 ppt but there is a significant reduction in scope for growth caused by the increased physiological stress at 16 ppt.

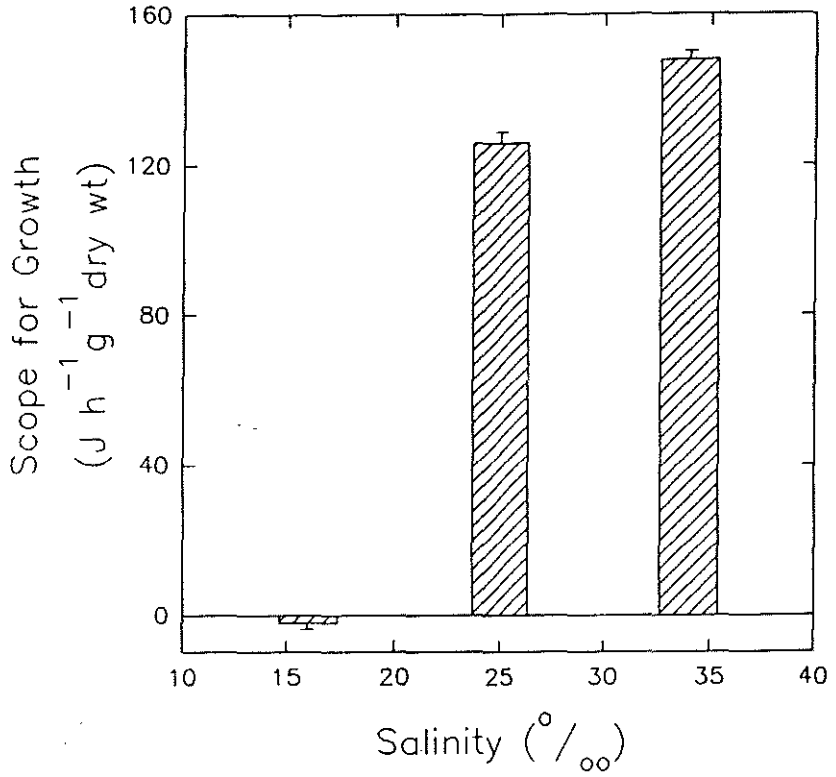


Figure 39. Scope for growth of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

The available results indicate that 34 ppt was the most favourable salinity for the stock of *O. edulis* that was studied.

4.2 METABOLIC EFFECTS (Figures 40-43)

As found with the temperature acclimation experiments the metabolic indices show variations in response to salinity that are concordant with the responses of the scope for growth indices. It was found that *O. edulis*

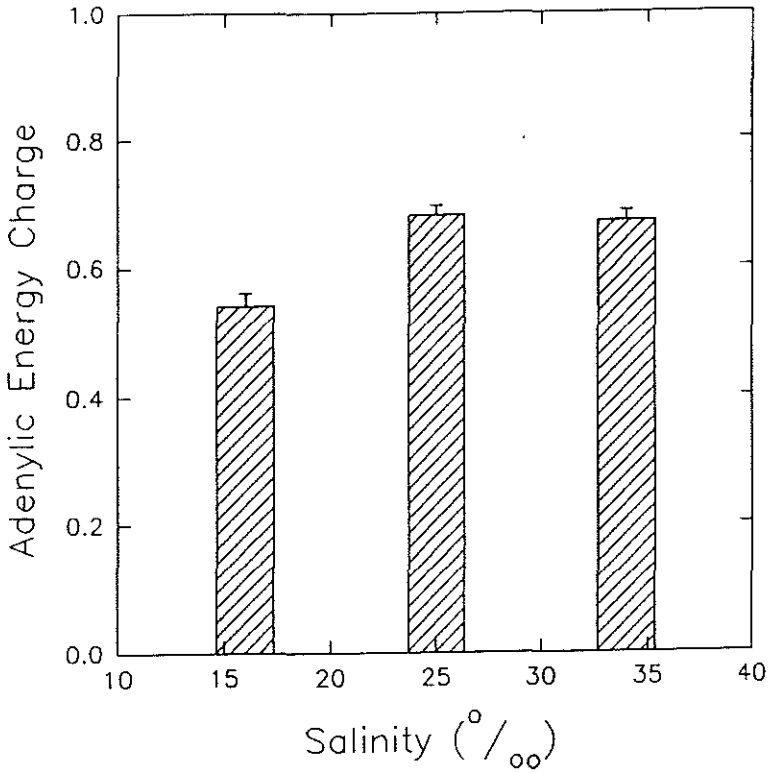


Figure 40. Adenylic energy charge of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

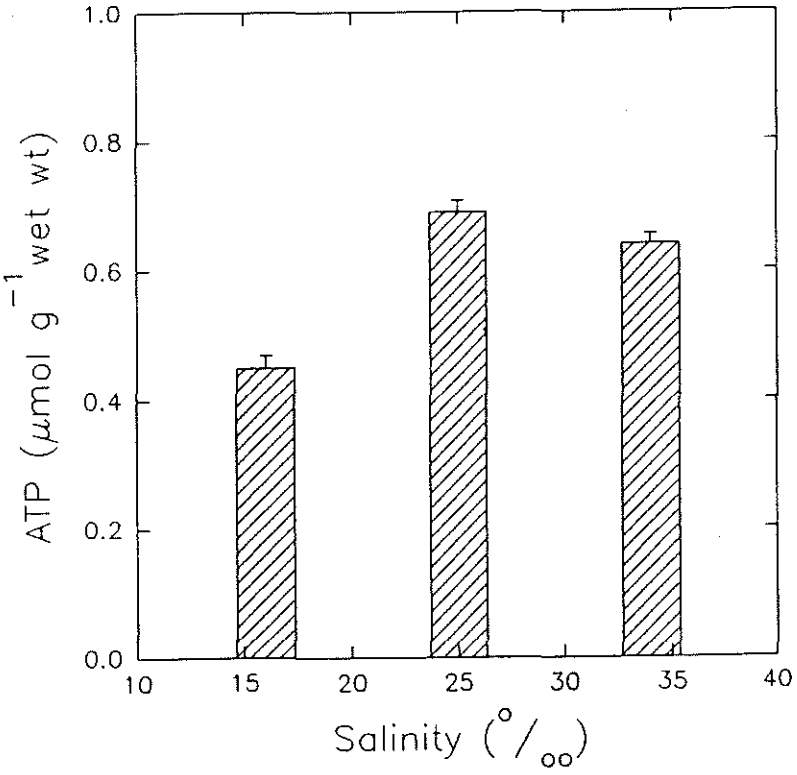


Figure 41. Adenosine triphosphate concentrations in the tissues of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

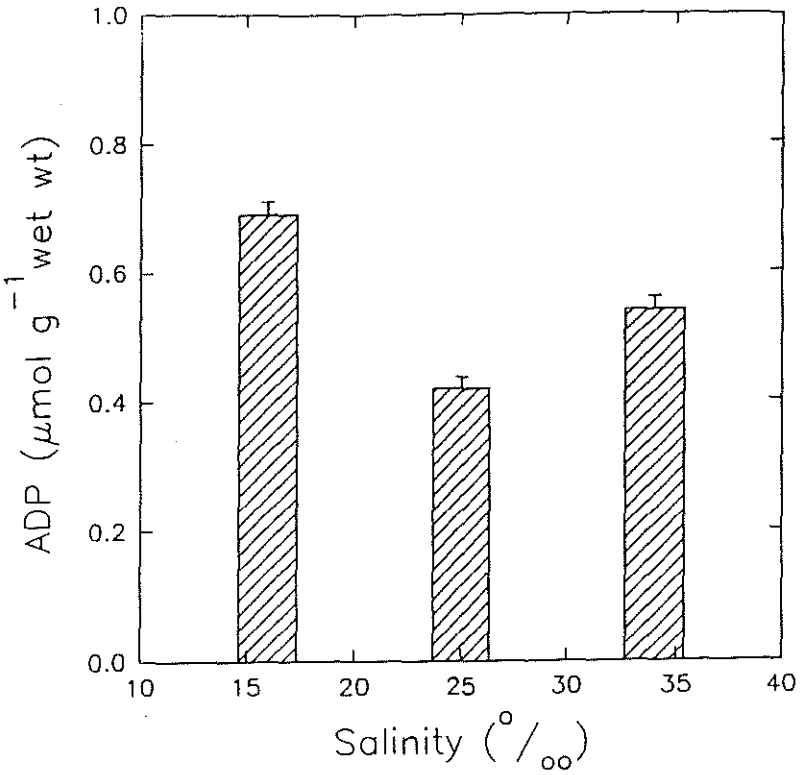


Figure 42. Adenosine diphosphate concentrations in the tissues of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

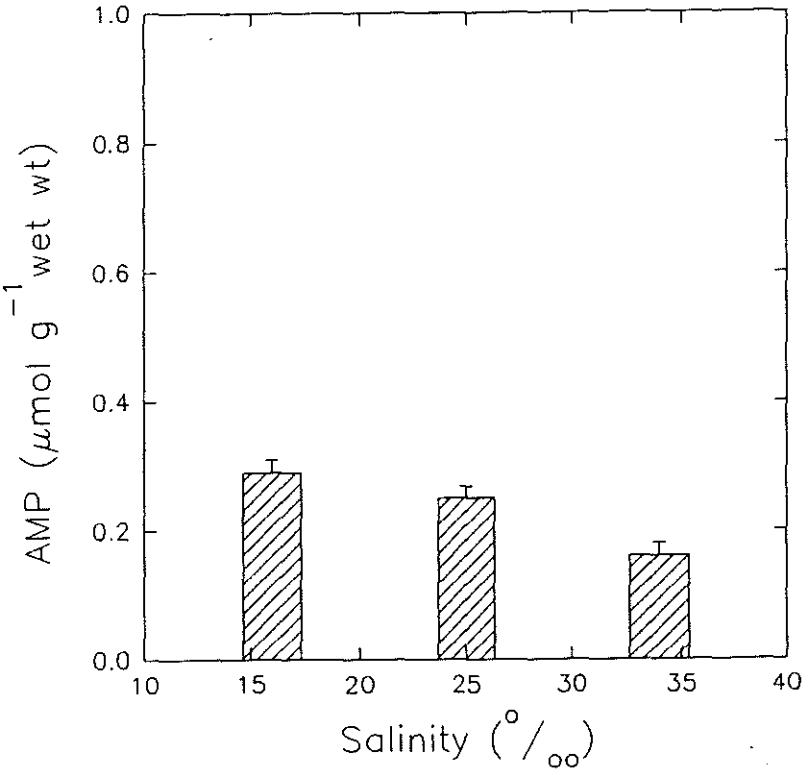


Figure 43. Adenosine monophosphate concentrations in the tissues of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

shows little response to salinity when it is reduced from 34 ppt to 25 ppt but at 16 ppt the accumulation of ADP and AMP implies that the rephosphorylation pathways do not function at maximum velocity under these conditions.

4.3 *HAEMOCYTIC* EFFECTS (Figures 44-51)

The responses of the amoebocyte locomotion rates is anomalous when compared to the physiological and metabolic responses to the lowest salinity of 16 ppt. Preliminary studies have found that amoebocytes taken from animals at this salinity are smaller and do not form the large aggregations that are seen in samples from animals at higher salinities. Thus, the increased rate of locomotion may be the result of the reduction to the resistance to movement caused by a loss of the adhesive properties if the haemocyte surface. Such a loss of surface binding capacity might be also expected to affect the ability of such haemocytes to attach to foreign particles and eliminate them by diapedesis.

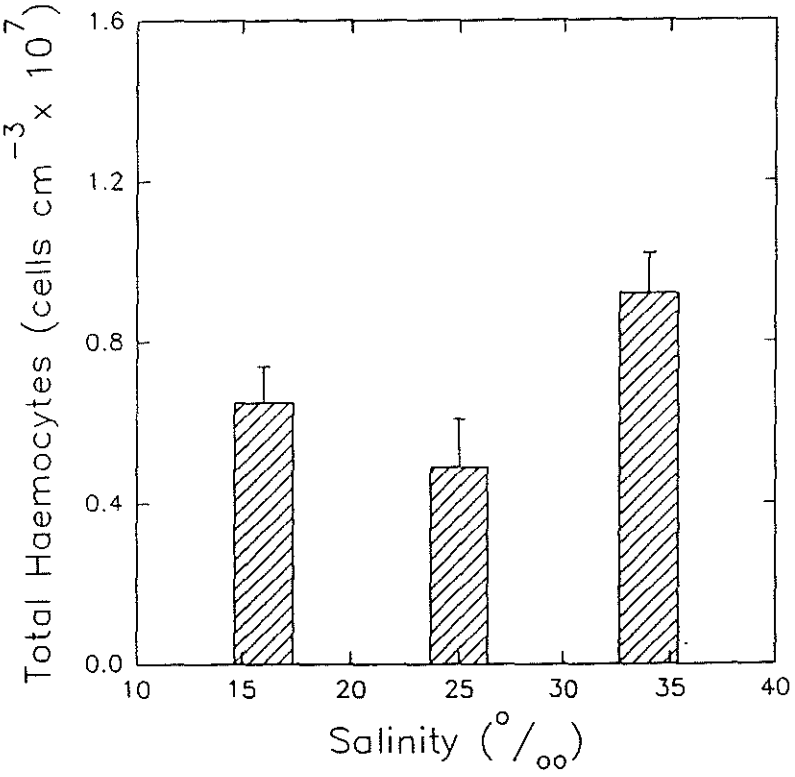


Figure 44. Total haemocyte concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

The percentage cell size change shows a linear relationship to salinity that is attributable to the cytoskeletal changes in the cell membrane induced by membrane permeability responses to the osmolality of the medium. Lysozyme concentrations are reduced at 25 ppt when compared to

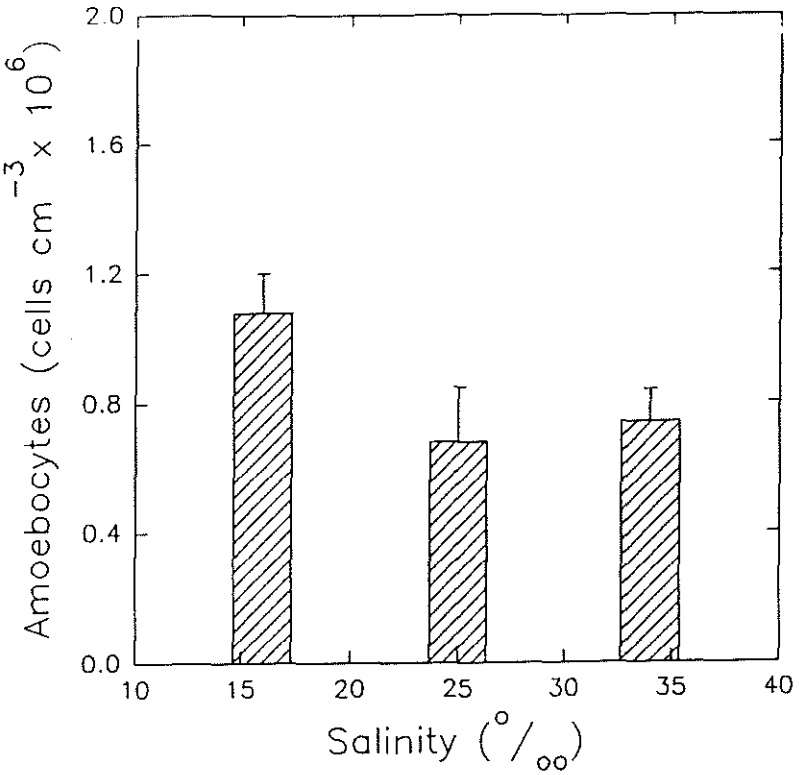


Figure 45. Total amoebocyte concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

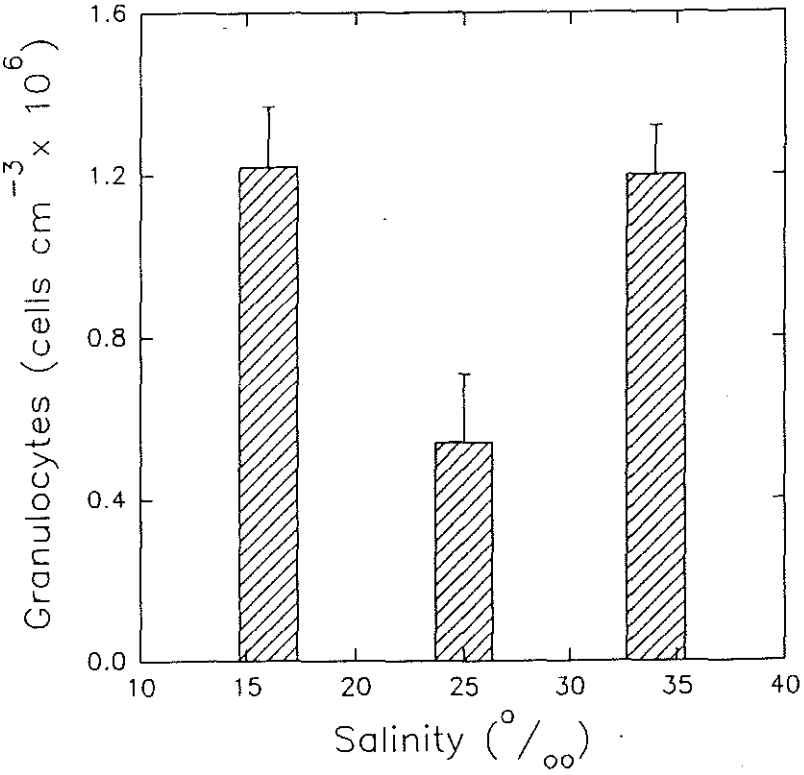


Figure 46. Total granulocyte concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

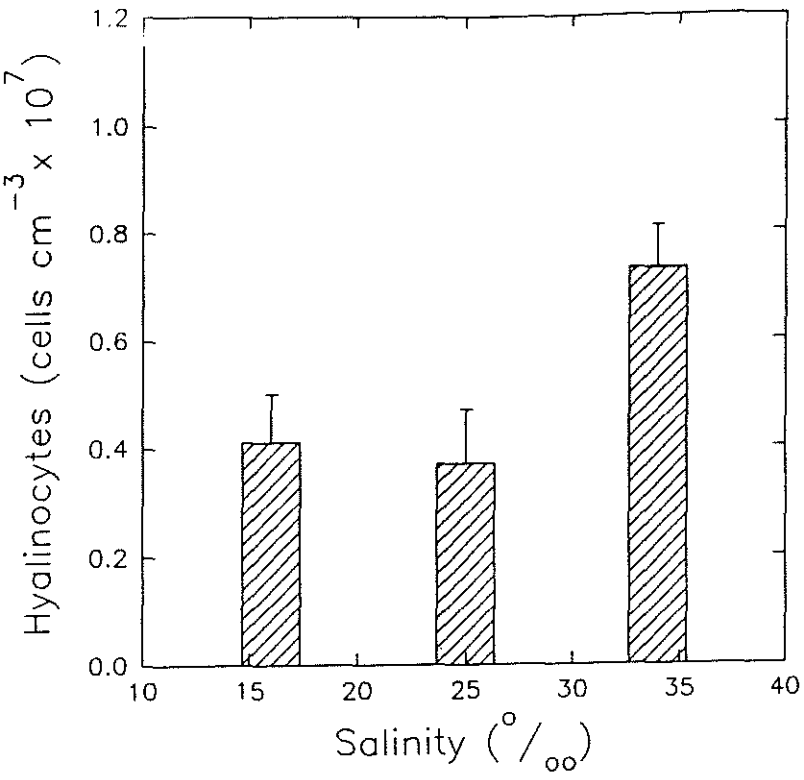


Figure 47. Total hyalinocyte concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

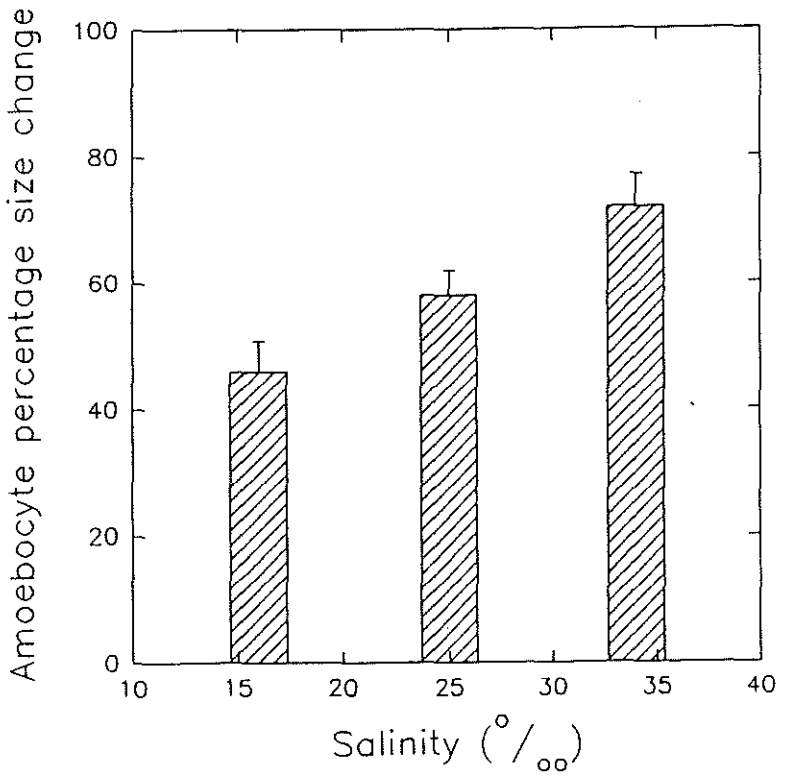


Figure 48. Amoebocyte percentage cell size changes of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

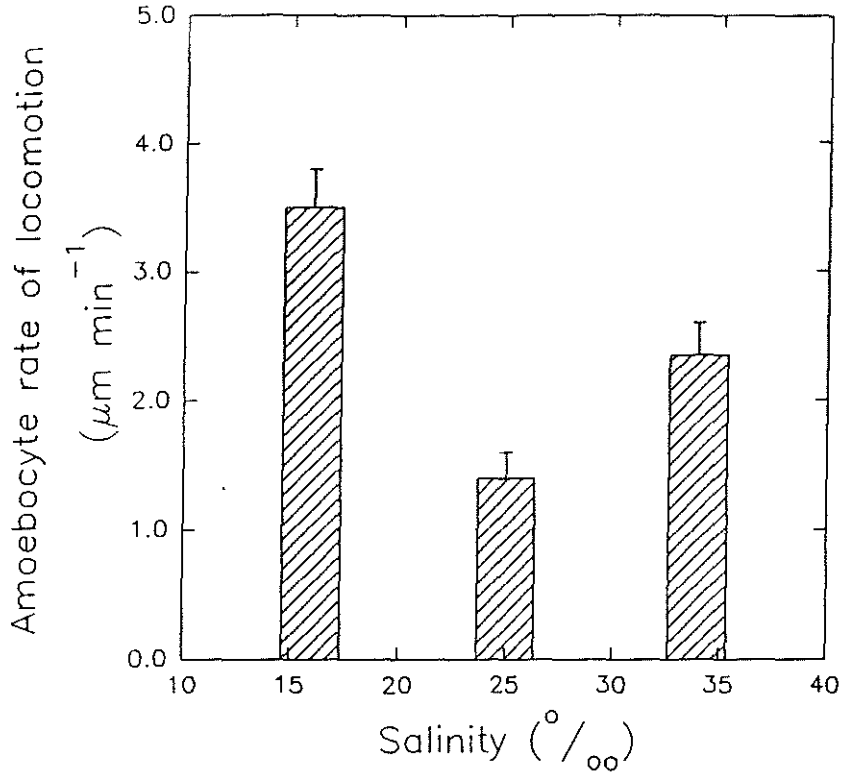


Figure 49. Amoebocyte rates of locomotion of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

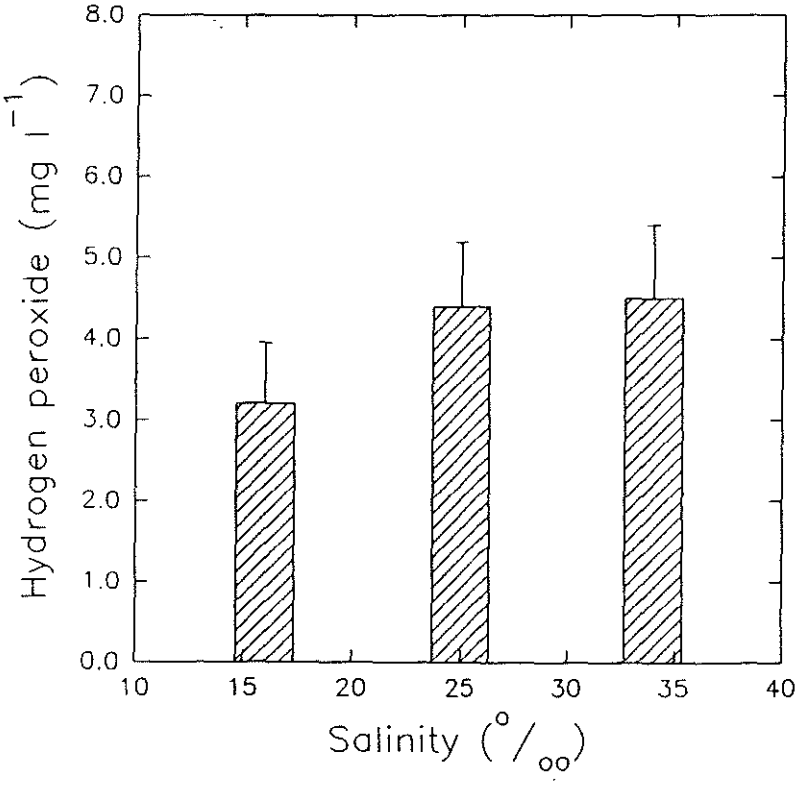


Figure 50. Haemolymph Hydrogen peroxide concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

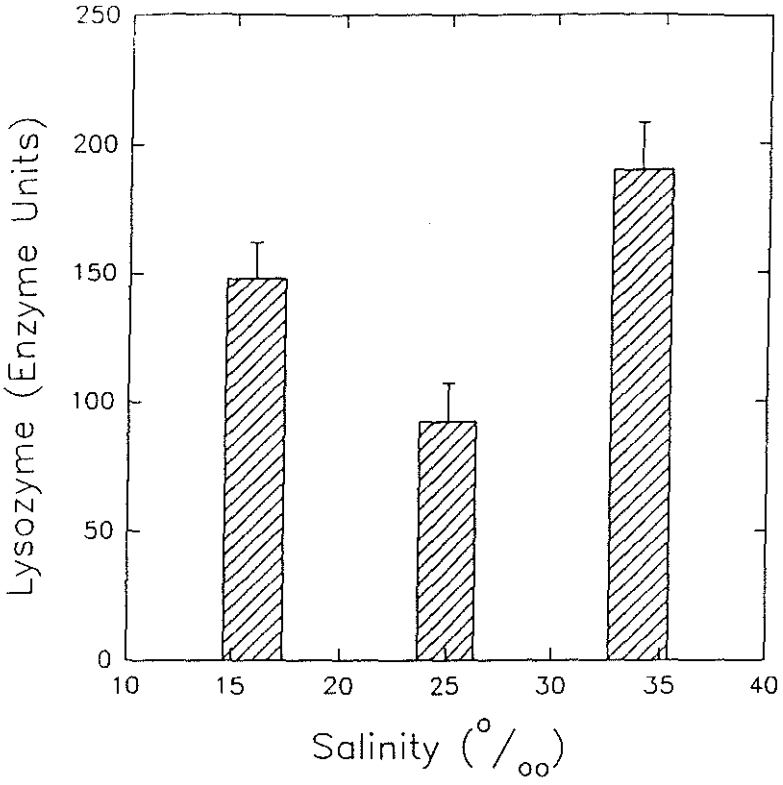


Figure 51. Haemolymph lysozyme concentrations of *Ostrea edulis* acclimated to 16, 25 and 34 ‰ and fixed temperature (15 °C).

those at 34 ppt but was found to be increased at 16 ppt. This increase may be attributable to the increased fragility of the cells at this salinity, T= 15°C (personal observation) causing a rapid turnover of cells releasing their storage products into the haemolymph. A rapid turnover of cells would also account for a reduction in the mean size of the amoebocyte population.

CHAPTER 5

THE EFFECTS OF TIDAL EXPOSURE

Quantitative assessments were made of the effects of stress on the haemocytes of *O.edulis* since these are the main cellular defences against pathogens such as *Bonamia* (61); lysozyme and hydrogen peroxide are produced as non-specific microcidal agents and haemocyte motility is a measure of the ability of the oyster to eliminate foreign material by phagocytosis and subsequent diapedesis. The integration of lysozyme, hydrogen peroxide and haemocyte motility measurements into the assays of metabolic stress effects provided direct observations of the exact relationship between the functioning of oyster defence mechanisms and the rest of the animal's life processes. In this way it was possible to bring together a number of previously disparate observations by incorporating them into a multi-system model of oyster responses to environmental stressors. For example, bivalve haemocytes can remain active during periods of anoxia such as tidal exposure (41) but also succinic acid is accumulated in preference to lactic acid under the same conditions (35). The accumulated succinic acid can be used by the haemocytes to sustain their activities whilst the rest of the tissues are quiescent but prolonged anoxia or high temperatures cause greater lactic acid production and a consequent reduction in haemocyte activity.

The investigation of the effects of tidal exposure are particularly relevant to the investigation of the vulnerability of *O. edulis* to *Bonamia* since the commercial exploitation of oysters has generally involved the cultivation of animals in shallow water where they are subject to tidal exposure as well as greater extremes of temperature, salinity and sediment load. It may be noted that the haemocyte-mediated defences of the hard clam Mercenaria mercenaria have been shown to be extremely sensitive to chronic tidal exposure, particularly hydrogen peroxide production (77,78).

The following results were obtained from animals taken from control and experimental groups acclimated to tidal change in water level of 1 metre over a 12 hour 25 minute cycle over a period of 60 days. The experimental group were placed in the experimental tank so as be exposed to air for 3 hours in every 12 hour 25 minute cycle. Animals were taken from both groups when the experimental group became exposed (time 0) and after 3 hours exposure (time = 3 hours). Water temperature and salinity varied over the ranges 14°-16°C and 33 ppt to 34 ppt. All animals were fed a mixed algal diet on the incoming 'tide'.

5.1 METABOLIC EFFECTS OF TIDAL EXPOSURE (Figure 52-53)

There was a rapid decline in the AEC on exposure as the ATP accumulated in the tissues during aerobic respiration was consumed. However, this decline was partially offset by the production of a small amount of ATP by anaerobic metabolic pathways. This form of metabolic adaptation has already been described by Hammen (35) in *Crassostrea virginica*.

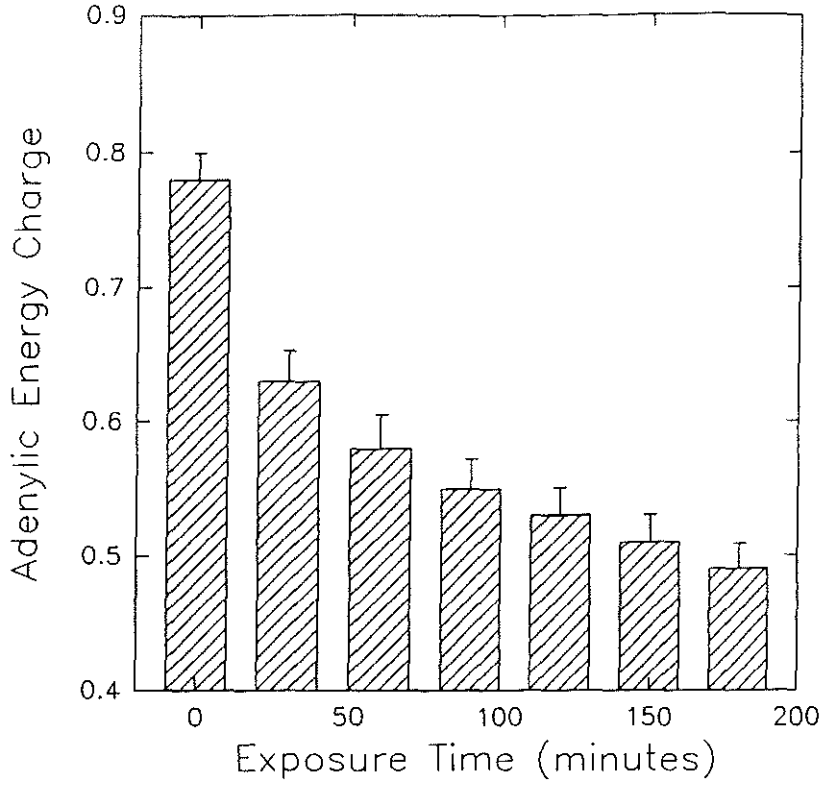


Figure 52. Effect of tidal exposure on adenylic energy charge of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S = 34 ‰).

The effect of chronic exposure stress on the differential generation of lactate and succinate is shown by Figure 53. The 3 hour exposure group show a substantial increase in both the absolute concentration and the proportion of lactate to succinate. This suggests that the generation of succinate by the truncated Krebs' cycle is more sensitive than the generation of lactate and ATP from pyruvate to decreasing oxygen tension and waste product accumulation during prolonged valve closure.

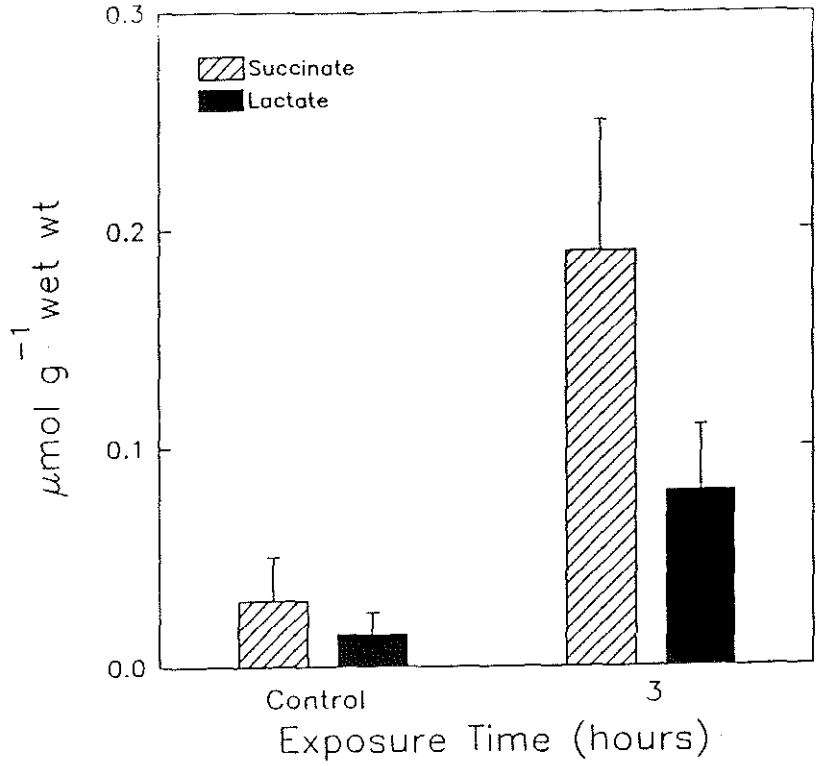


Figure 53. The effect of tidal exposure on tissue lactate and succinate concentrations of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S = 34 ‰).

5.2 HAEMOCYTIC EFFECTS OF TIDAL EXPOSURE (Figures 54-61)

Haemocyte numbers were maintained at approximately constant levels in the control group whereas the numbers of haemocytes circulating in the haemolymph of experimental groups were, unexpectedly, greater than the control at time 0, before dropping to a concentration below that of the control value.

Tidal exposure appeared to cause reduction in amoebocyte numbers in the experimental group when compared to the control group; this reduction was more pronounced after 3 hours exposure.

As has been noted in the second interim report variations in granulocyte numbers very closely followed variations in amoebocyte numbers in response to salinity and temperature. However, this relationship was not so apparent with respect to tidal exposure though the exposed group showed a significant reduction in granulocyte numbers after 3 hours exposure.

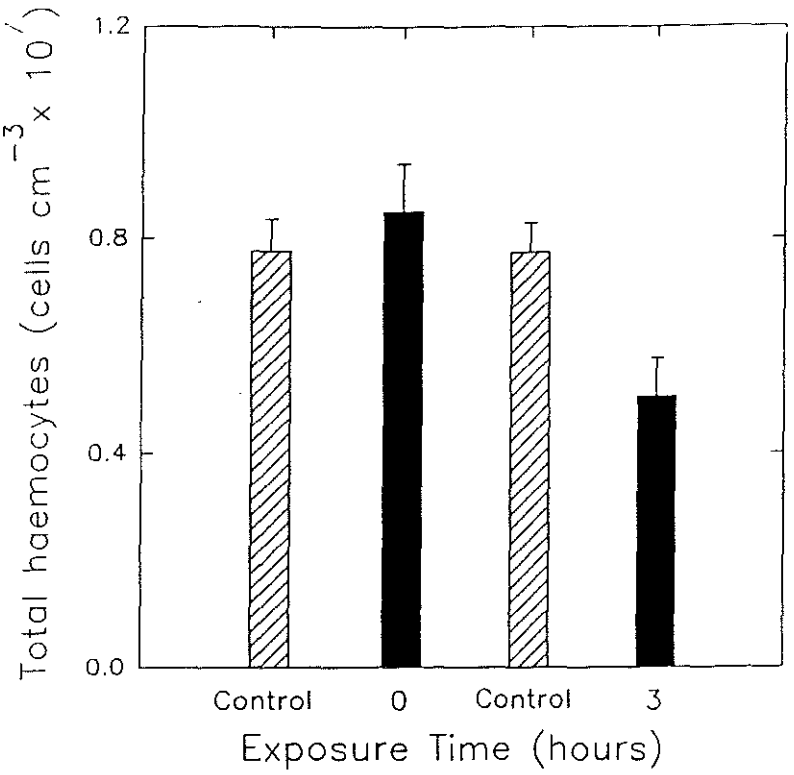


Figure 54. Effect of tidal exposure on total haemocyte concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

Hyalinocyte numbers show the same pattern of changes shown by the other two types of haemocyte but the increase in hyalinocyte numbers did not produce a corresponding increase in hydrogen peroxide, unlike the relationship between amoebocyte numbers and lysozyme concentration.

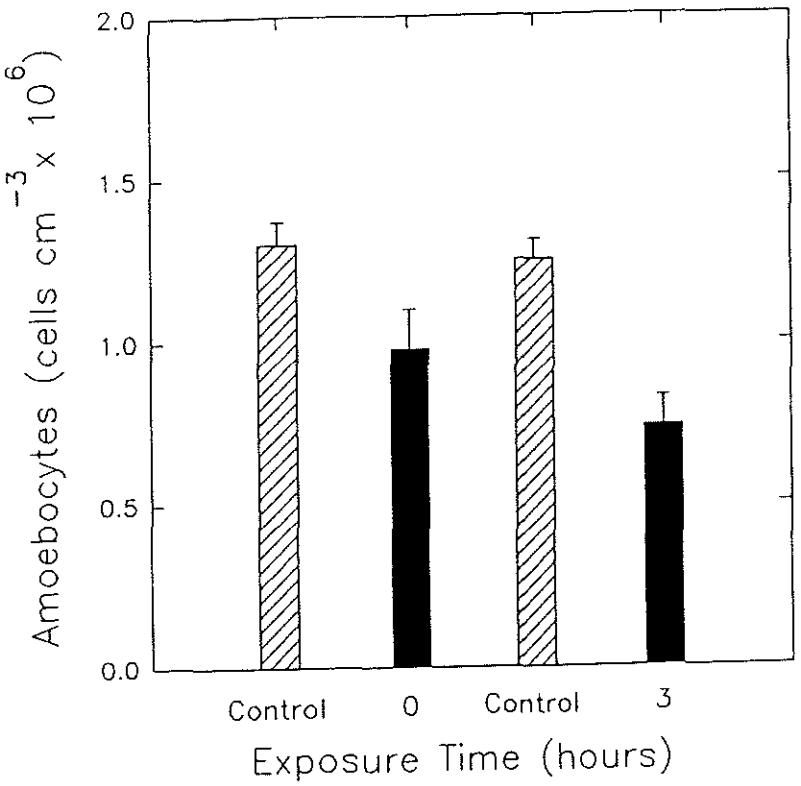


Figure 55. Effect of tidal exposure on amoebocyte concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

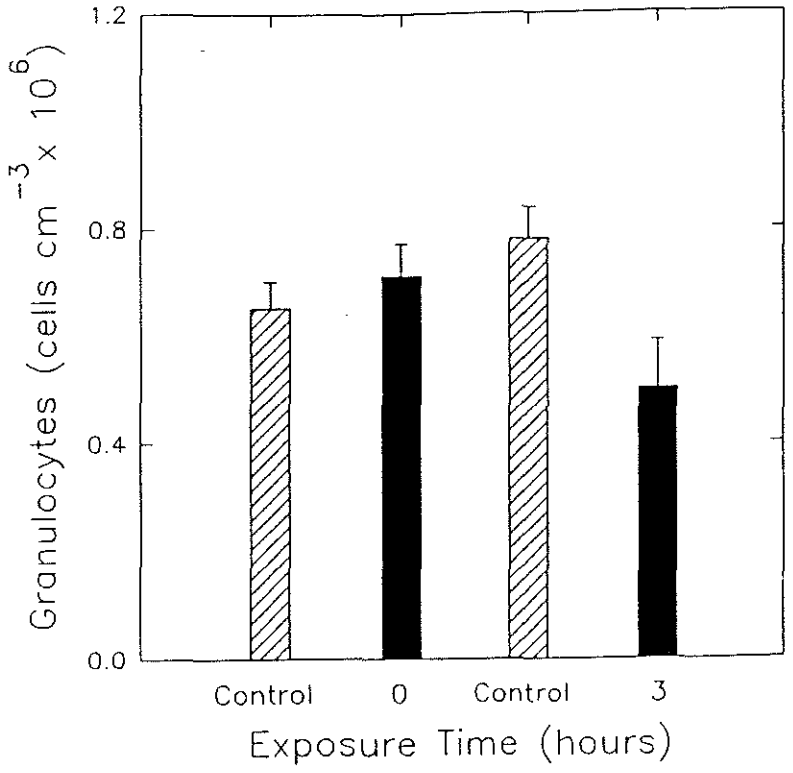


Figure 56. Effect of tidal exposure on granulocyte concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

Control values remained approximately constant during the tidal cycle whereas exposure reduced the haemolymph hydrogen peroxide titre compared to the control group.

The control group lysozyme concentrations showed no significant change in during the experimental period. In contrast, the 3 hour exposure group had slightly greater lysozyme concentrations after three hours exposure. It may be noted that reductions in amoebocyte numbers were coincident with lysozyme maxima in both groups suggesting that lysozyme release is related to the loss of amoebocytes.

During the course of the exposure phase the amoebocyte motilities of the control group remained unaltered. However, the motilities of amoebocytes from the experimental group declined on exposure. The ability amoebocytes to increase in size remained approximately constant in the control group but was greater in the experimental groups, although there was rapid decrease in the cell size changes after exposure coincided with the diminution of cell motilities.

On exposure, the animals respiration becomes anaerobic, as the valves clamp tight shut. The lack of oxygen means the Krebs Tricarboxylic Acid cycle can no longer be utilised, and instead lactate is produced. Small levels of this metabolite cause a slight decrease in pH which causes a switch in the animals enzyme system; this reverses the latter part of the TCA cycle causing a build up of succinate.

The production of succinate has shown to be proportional to the length of tidal exposure, whereas lactate production is initially low, but as the period of anaerobia continues, its concentration increases substantially, as does the proportion of lactate to succinate. The accumulation of succinate rather than lactate has a number of advantages for the oyster, including :

1) Succinate is a weaker acid than lactate, and so requires less buffering by ions in the mantle fluid. Its weakness also means that it is less toxic to the oysters tissues.

2) When aerobic respiration resumes, the conversion of succinate to oxaloacetate via the TCA cycle yields a net gain of ATP, whereas the oxidation of lactate uses the same amount of ATP as was produced when the lactate was building up.

The proportional quantities of these metabolites has an affect on the haemocytic state of the exposed animals, and particularly on the amoebocytic movement and ability to function as a phagocytic defence mechanism.

Previous work has shown the effect of other environmental variables such as temperature and salinity on rate of amoebocyte locomotion and percentage size change. Rate of movement changes proportionally with temperature, and shows a drop then increase with decreasing salinity. The size change is slowest at low temperatures, highest at ambient temperatures, and decreases proportionally with decreasing salinity. Combinations of these variables have shown that optimal conditions occur at middle range salinities, and 15°C (ambient temperature in laboratory conditions); high temperatures and low salinities, as are often encountered in summer storms in shallow waters, have proved to be lethal.

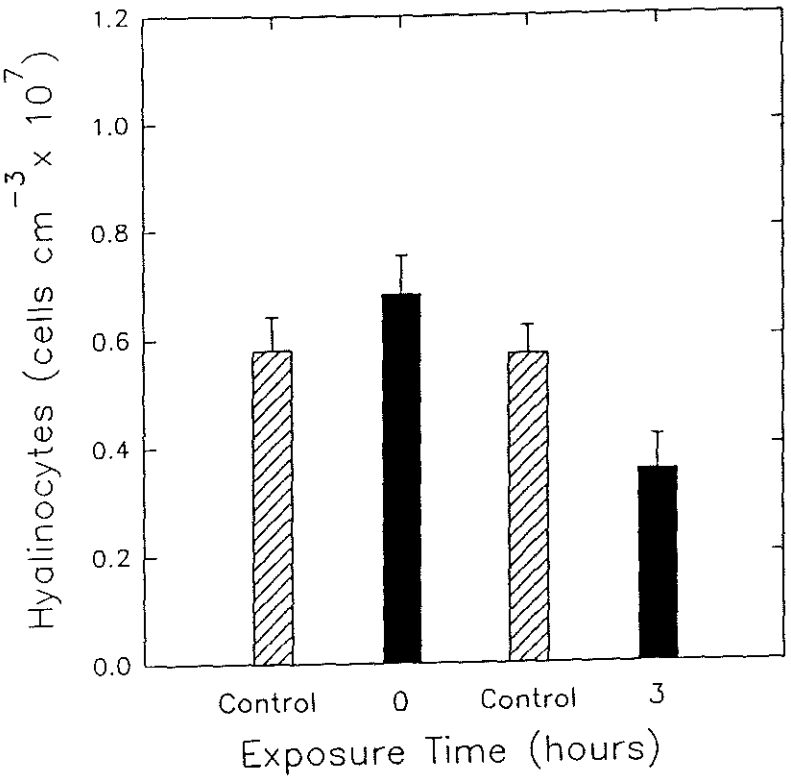


Figure 57. Effect of tidal exposure on hyalinocyte concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

OBSERVATION	NORMAL CELLS	+ LACTATE
pH	7.3-7.5	7.3-7.5
Cell aggregates	Separate	Remain Aggregated
Pseudopodia	Present	Disappear after 5 minutes
Rate of locomotion (μ min^{-1})	5.5 ± 0.3	0
% Size increase	62 ± 13	7.3 ± 3.6

Table 9: The effect of lactate exposure on *in vitro* amoebocyte activity.

Under conditions of tidal exposure, the motility of amoebocytes has been shown to be inversely proportional to the length of exposure, and that the maximum motility of amoebocytes in exposed groups occurs during the exposure phase, but becomes curtailed with time. However, the effects of the accumulation of metabolites on the behaviour of amoebocytes has not been shown.

Measurement of the pH of the mantle fluid and of the lactate sea water for the challenge showed that they were not significantly different, which shows that any changes in amoebocyte activity were NOT as a result of pH change. This is very important, suggesting that the change is a result of metabolite concentrations - in this case it must be because of the lactate concentration. Also, cells of the same animal have been compared, so the differences in behaviour are due to differences in treatment and not as a result of inherent traits.

The effect of lactate is initially one of suppression of movement, with no observed separation of clumps of amoebocytes. These cells still project pseudopodia at first, although single amoebocytes become quickly rounded. After 5-10 minutes, the clumps of amoebocytes withdraw their pseudopodia, and show rounding up. Some of the oysters sampled were coming into breeding condition, and sperm motility was also observed to be greatly reduced in the presence of lactate.

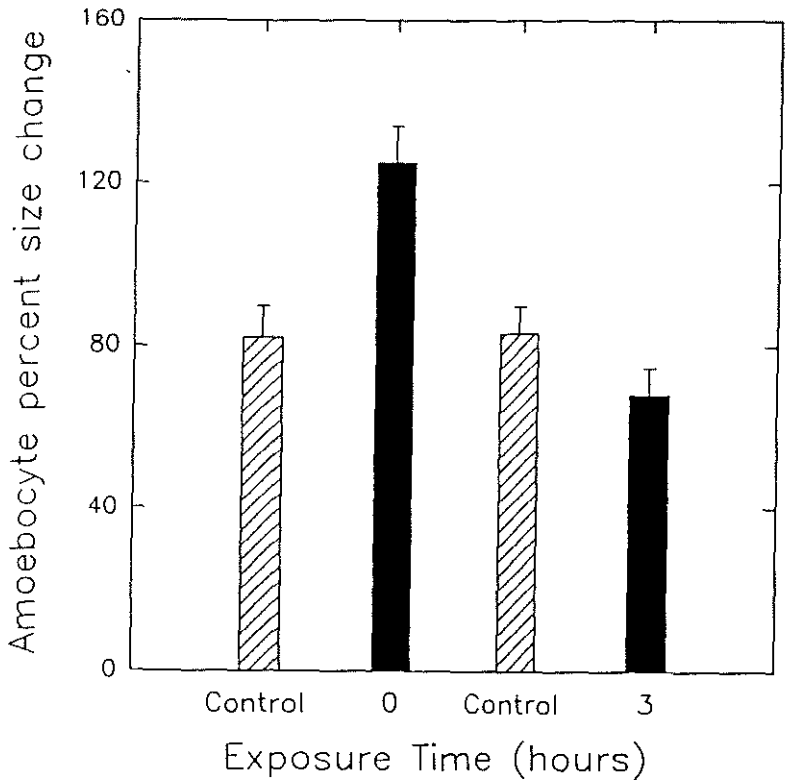


Figure 58. Effect of tidal exposure on the percentage size change of amoebocytes of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

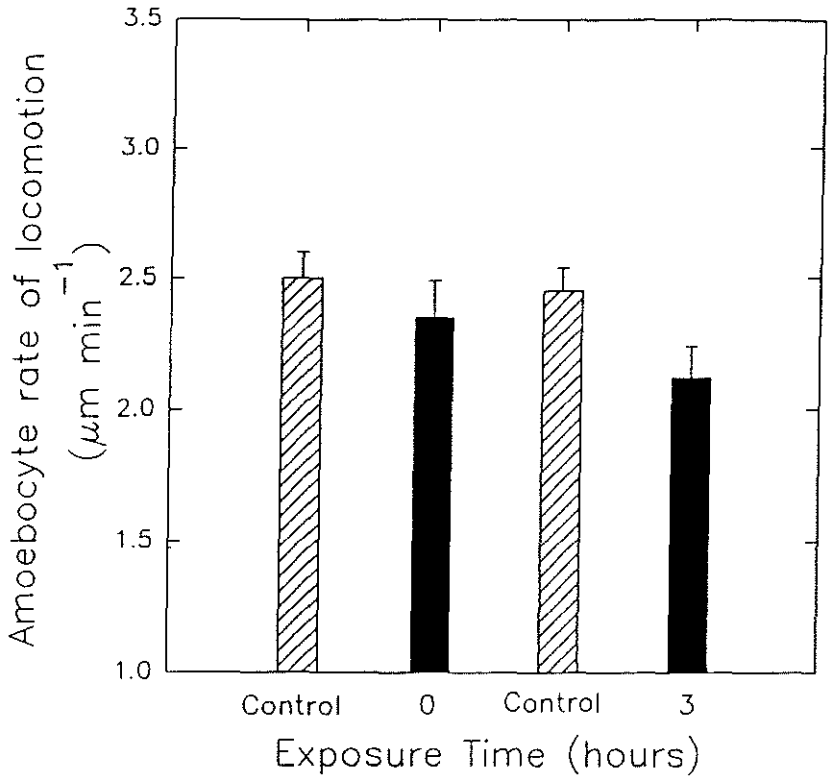


Figure 59. Effect of tidal exposure on the rate of amoebocyte locomotion of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

The condition of the oyster must also be considered when making observations and assumptions about the animal. The onset of its "summer physiological state" is triggered by increasing temperature, and results in its coming into breeding condition. The production of gametes involves the diversion of energy from the animals defence mechanisms, which leaves the animal prone to infection.

However, from these results, it shows that changes in motility and size are as a result of lactate concentration which is in turn a product of anaerobic respiration due to prolonged periods of tidal exposure. These effects on the phagocytic capabilities of the amoebocytes serve to seriously affect the defensive ability of the animal, leading to an increased disease susceptibility.

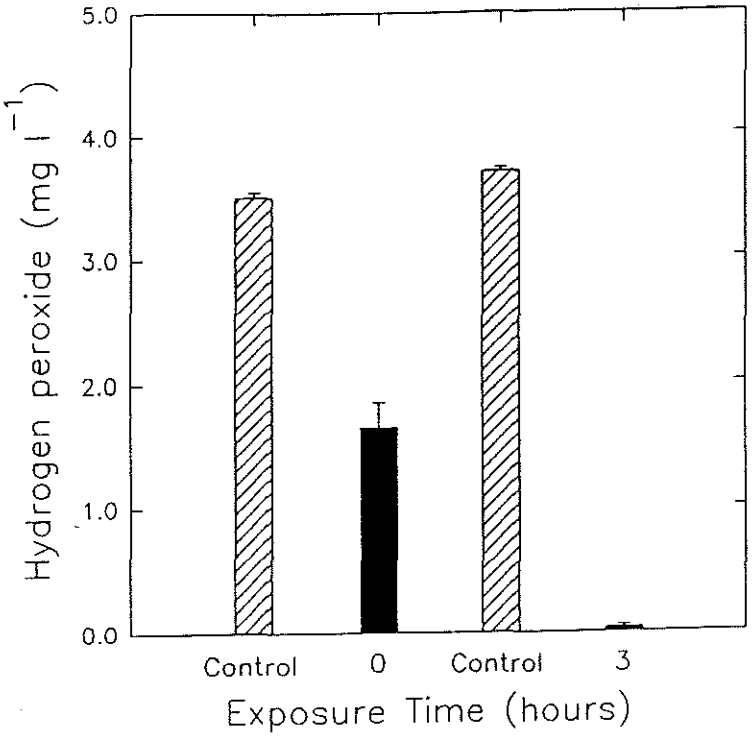


Figure 60. Effect of tidal exposure on haemolymph hydrogen peroxide concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

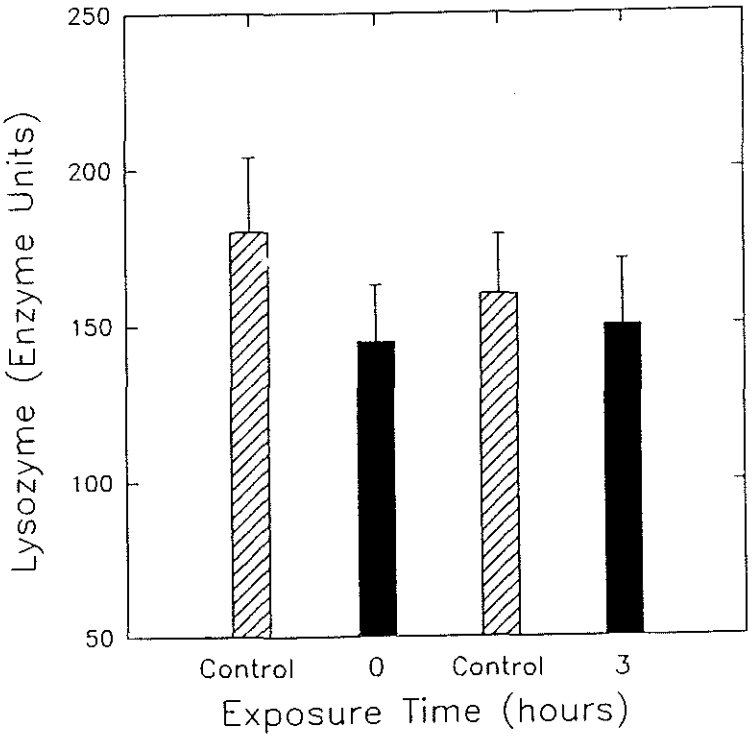


Figure 61. Effect of tidal exposure on haemolymph lysozyme concentration of *Ostrea edulis* exposed to an air temperature of 15 °C (Water temperature = 15 °C, S=34 ‰).

CHAPTER 6

DISCUSSION

When considering exposure as a stressor, there are a multiplicity of factors and processes within the animal which are affected. These cannot be considered independently of one another, and it is important that the whole picture is analysed before conclusions are drawn. Thus, the contributions of the three groups of stress indexation methods are considered first, individually but the information gained from the summation of all three approaches is combined into a holistic view of the environmental aetiology of bonamiasis.

The Scope for Growth index (SFG) has been used widely as a relatively simple means of detecting sub-lethal stress in marine organisms; it has been most commonly used to quantify the responses of marine bivalves to environmental stress. Its use in the present study has proved useful in the construction of a hypothesis seeking to explain the relationship between disease susceptibility and environmental conditions. However, the sensitivity of the method to food input creates a number of disadvantages. Firstly, the zero SFG value in relation to a response curve for a given physico-chemical variable can moved up or down by changing the food ration, this means that results from different sites or experiments may not be comparable. Secondly, the method is heavily influenced by intrinsic metabolic characteristics of

the study organism that depart from the assumption of perfect poikilothermy; in *O. edulis* interpretation of SFG must take account of the temperature-related hysteresis in filtering rate and the switch between `winter' and `summer' physiological states. Thirdly, the method cannot be applied in all marine environmental conditions - the SFG's of marine organisms cannot be measured during air exposure. These considerations have necessitated the use of biochemical indices to determine the metabolic basis of the SFG results.

Biochemical indices are able to provide a great amount of detailed information on the metabolic responses to environmental stressors, resolving subtle changes that take place over periods covering time scales from months, such as gametogenesis and brooding, to transient changes that take place over a few minutes such as the changes in AEC during tidal exposure. However, these advantages have to be weighed against the much greater complexity of the analytical techniques, although the development of the integrated assay procedure has made analysis times comparable with the physiological and biometric indexation methods. Biochemical stress indices are relevant to the investigation of disease susceptibility since metabolic activity will directly influence haemopoiesis, anabolic pathways synthesising humoral defences such as lectins and the activity of cellular defences neutralising and removing potential pathogens by diapedesis.

The results that were obtained from the biochemical assays have helped to define the optimum and stressful conditions and shown evidence of the metabolic switch between `winter' and `summer' metabolic states. This phenomenon has not been previously described in *O. edulis* but which Fisher & Chintala (79) have shown to be important in the immunological responses of another oyster species *Crassostrea virginica*. This and the other recent studies of *O. edulis* have also confirmed for the first time that *O. edulis*

possesses the same adaptation to anaerobic respiration as a number of other bivalves i.e. the accumulation of succinate in preference to lactate, and the breakdown of this mechanism under chronic and prolonged stress. This metabolic change will affect disease susceptibility by the reduction of haemocyte activity as body fluid pH increases and the reduced oxygen tension combined with accumulated succinate may create conditions favouring the proliferation of pathogenic microorganisms.

The integration of assay methods to give simultaneous measurements of the haemolymph titres of microcidal substances, haemocyte activity and metabolic biochemistry from the same animal has demonstrated interrelated responses to abiotic variables. This pluralist approach has also been shown to be effective in studying the effects of biological phenomena in the form of the phytoplankton bloom succession and associated elevations of microbial load (80). Observations of immunological functions also reveal the relationship between disease susceptibility and intrinsic factors, particularly reproduction. The suppression of the production of microcidal agents, whilst retaining amoebocyte activity during larval brooding, was an unexpected aspect of reproductive stress, suggesting that there was some form of recognition system in operation to prevent brooding larvae from inducing an inflammation reaction within the mantle cavity. The data from the temperature shock experiments made a significant contribution to the understanding of the vulnerability of re-laid oysters to infection and the observation of the severe reduction or abolition of amoebocyte activity at temperatures $\geq 25^{\circ}C$ was of particular interest. The increase in presumptive disease susceptibility not only arose from the inhibition of amoebocyte activity but also from the loss of these cells which is caused by their fragility and autolysis at high temperatures this is also reflected in the reduction in numbers and size of subsequent samples.

The observations of the variations in the numbers of the three haemocyte types has contributed to the still unresolved debate on the relationship between the haemocyte types in marine bivalves. The paralleling of amoebocyte numbers by granulocyte numbers supports the dual cell line hypothesis of Balouet & Poder (81) and Cheng (82). These workers have proposed that the hyalinocytes form one lineage and the granulocytes are an intermediate or immature stage in the lineage culminating in the formation of amoebocytes. Monitoring changes in relative and absolute numbers of haemocytes is an obvious quantitative index of immunological status that can be carried out quickly and simply.

The quantitative examination of haemolymph and haemocyte characteristics can be used as direct measures of immune function but there are certain inherent practical difficulties associated with these methods. The main problem that has been encountered has been the great variability in the cell counts between individuals from identical conditions; this phenomenon has also been commented on by other investigators studying *O. edulis* - Chagot (44) found variations close to an order of magnitude in samples of 50 individuals. This probably represents the variability of response that is innate in natural populations and necessary for their survival (83,84). Also, the present study has shown that the apparent numbers of haemocytes are extremely labile, as exemplified by the rapidity of the change in apparent numbers during tidal exposure (Figure 54). It is this type of observation that has vindicated the adoption of the pluralist methodology to facilitate the verification of results from independent techniques and has led to the formulation of a whole animal hypothesis of the relationship of disease susceptibility to environmental stress.

When the results described above are taken in conjunction with information from related work (85,86) it is possible to construct a hypothesis which seeks to explain some of the common factors associated with the occurrences of bonamiasis in European waters. The principal features of bonamiasis were firstly, the very marked prevalence of infected animals and subsequent mortalities at shallow water sites where levels of infection could be positively correlated with the degree of tidal exposure. Secondly, the most severe losses tended to occur in areas of intensive ostreiculture particularly where there were frequent movements of oysters for re-laying. Any natural stocks that remained unmoved showed much less mortality and lower levels of infection.

It is possible to explain two aspects of the occurrence of *Bonamia* related mortalities in terms of the effects of the environmental stressors on the ability of *O. edulis* to resist this (and other) pathogens. Firstly, the seasonal variation and prevalence of the disease in shallow waters, where mortalities become noticeable in spring and rise to peak in early summer. Secondly, the very much greater susceptibility of re-laid stock compared to any endemic population of the same species in the same area.

Animals in harbours, estuaries and creeks used as re-laying grounds are subject to large variations in temperature and salinity. In winter water temperatures fall quickly, inducing the `winter' physiological state whilst the increase in freshwater inputs will lower salinity. The metabolic and immunological indices have shown that temperatures below 10°C enable salinities as low as 16 ppt to be tolerated whilst the immune system remains fully functional. However, in spring the water temperature in shallow waters rises rapidly and gametogenesis will be initiated, diverting energy from the maintenance of the immune system. At the same time the transition to the `summer' state at temperatures above 10°C renders the immune system increasingly vulnerable to salinity stress; amoebocyte activity can be completely abolished by a combination of high temperature and low salinity that can be easily generated by summer storms. It is interesting to note that the occurrence of *Bonamia* in British waters coincided with a series of wet summers in the mid 1980's. Disease susceptibility is also increased during the larval brooding period in late spring and early summer when there seems to be partial suppression of the immune system whilst late stage larvae are held within the adult shell. By mid-summer water temperatures in shallow water can exceed 25°C, causing a loss of amoebocytes by heat-induced autolysis and reducing the motility of remaining cells. Animals that are subject to tidal exposure suffer even greater stresses so that the mortalities in these areas are often much larger.

The differential disease susceptibility of endemic and re-laid oyster stocks can be explained in terms of the effects of stressors on the functioning of the enzymes controlling metabolic processes and, in particular, those directly controlling haemocyte functions; this is a logical development of Atkinson's (33) `key enzyme'model of metabolic regulation; isozyme-based differences in the ecophysiology of oyster species are well-known and have been reviewed by Koehn (87). Under normal conditions stocks in deep water such as the oyster beds in the Solent are not subjected to tidal exposure or rapid changes in temperature and salinity and possess an isozyme complement suited to the relatively stable conditions. By contrast, natural stocks in shallow water have metabolic adaptations such as increased arginine phosphate and succinate accumulation to cope with tidal exposure (85) and isozymes that are insensitive to

temperature and salinity extremes. The haemocytes of the shallow water animals are therefore likely to function under conditions not normally encountered by animals from deeper water. When oysters are removed for re-laying they are usually taken from deeper water and subjected to prolonged air exposure i.e. prolonged shell closure which causes severe anoxia. This is often coupled with temperature shock which it is now known to cause the inactivation of the haemocytic defence mechanisms, probably by the inactivation of their metabolic enzymes. These immunologically compromised oysters are then placed in waters in which they might eventually be able to make use of the increased food available but they are also subject to increased environmental stress which will tend to delay the recovery of their defence systems. Thus the re-laid oysters are susceptible to pathogens that would be eliminated or controlled by fully functional haemocytic defences such as those of the endemic stock.

PART IV

STUDY OF THE VARIABILITY OF SOME HAEMOLYMPH PARAMETERS IN RELATION TO THE DISEASE

S. CULLOTY, A. FIGUERAS and M. MULCAHY

CONTENTS

CHAPTER 1

INTRODUCTION

The composition of the haemolymph of marine bivalves, has been reported by many authors indicating the presence of ions, enzymes, nitrogenous compounds, agglutinins and proteins... (reviews by 82;88;89;90;91;92). The determination of enzyme activity levels in mammalian blood has been often a useful diagnostic tool for disease. Enzyme leves outside the normal or reference range are regarded as a sign of tissue damage. The haemolymph of molluscs is involved in functions including defence and nutrition. So, its component levels may reveal the effects of any pathological or environmental parameters on the animal. Seasonal and environmental influences on the haemolymph composition have been known with a relationship to the reproductive cycle (42;93). On the other hand, several studies have documented changes in serum composition associated with parasitism. Decreased concentrations of lysozyme (94) and serum protein (95;96) in oysters have been correlated with *Haplosporidium nelsoni* (MXS) infection. In the same way, Mulvey and Feng (97) have observed alterations in haemolymph protein concentration in mussels infected with *Protoeces maculatus* . In contrast, Chu and La Peyre (93) did not found any correlation between oyster haemolymph lysozyme and protein levels and parasitism by *Perkinsus marinus*.

The present study is an attempt to study the relationship between mortality rates, *Bonamia* infection and some haemolymph components of the flat oyster *Ostrea edulis*.

CHAPTER 2

MATERIAL AND METHODS

2.1 ANIMALS

Oysters were obtained from Ireland and from Spain.

Hatchery-reared spat obtained from oysters from the Ria de Vigo in Spain, was cultured. After 12 months of age, this stock was separated in three clearly distinguishables length groups. Each group was placed in baskets on rafts at Domayo (inside the Ria de Vigo). From March to December 1991, 20 oysters were collected monthly. Mortality was estimated by counting the number of dead and live oysters from the total from each group.

For the haemolymph component study samples were taken from by oysters placed at two sites in the Ria de Vigo (NW, Spain): Limens (outer area) and Domayo (inner area). The experiments were conducted over a one year period and 20 oysters from each locality were collected in Spring (April), Summer (July), Autumn (October) and Winter (January).

In addition, oyster haemolymph ion levels were measured over a 28 day period in two groups of oysters from Ireland: 1) oysters which had been inoculated with purified *B. ostreae*, 2) controls inoculated with filtered seawater.

2.2 HAEMOLYMPH COMPONENTS

They were subsampled for analysis of serum components and cell numbers determined with a "Neubauer" chamber.

2.2.1 Ion and enzyme levels

The haemolymph samples were withdrawn with a syringe from the adductor muscle of the flat oysters. 200 to 600 µl of haemolymph was removed from each oyster and spun on a bench top centrifuge. Measurement of ion and enzyme levels was carried out in a selective multichannel autoanalyser (Hitachi, Model 737). Constituents originally analysed included: total protein, calcium, creatinine, urate, sodium, potassium, chloride, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase (Asat), lactate dehydrogenase, glucose, cholesterol, bilirubin and protoporphyrin.

Haemolymph for ion measurements was sampled from oysters that had been inoculated with purified *Bonamia*.

Amylase, aspartate aminotransferase (Asat) measurements were carried out on monthly samples obtained from 1988 oysters.

2.2.2 Proteins

Protein measurement was carried out by following a procedure to measure human urinary protein (Watanabe et al., 1986). Results are based on a colour reaction produced between a pyrogallol red-molybdate complex and protein. Human albumin (Sigma) of varying concentrations was used to produce a standard calibration curve from which protein levels were read.

For Spanish oyster samples, protein was estimated by the classical method of Lowry *et al* (1951) with bovine albumin serum as a standard.

2.2.3 Lysozyme

The turbidimetric assay for lysozyme (98) was performed with 0.15 mg/ml lyophilized *Micrococcus luteus* (Sigma) dissolved in 66 mM sodium phosphate buffer (pH 6.2). The reaction was carried out at 22°C and the absorbance at 450 nm measured after 5 and 10 min. The unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed decrease in absorbance of 0.001 min.

2.2.4 Agglutinins

The assay of PLP (Polystyrene Latex Particles) agglutinin titer was performed with serum serially diluted in a 96 cell-well plate and PLP (2.5%). The mixtures were incubated at room temperature. The titers will be determined by comparing with the control visually.

2.2.5 Histological examination

In both studies, the sample animals were weighed and after shell removal all tissues were fixed in Davidson's fixative. An anterior transverse section was taken that included the digestive gland, mantle and gills. Histological sections were cut 5µm thick and stained with iron haematoxylin, acid fuchsin and aniline blue.

The gametogenesis was studied by measuring the relative volume fractions of vesicular connective tissue cells, developing and ripe gametes.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 CONSTITUENTS DETECTED IN THE AUTOANALYSER

Calcium ($Ca2^+$), sodium (Na^+), potassium (K^+), chloride (Cl^-) ions, amylase and aspartate aminotransferase (Asat) were the only constituents that registered activity on the selective multichannel autoanalyser.

3.1.1 Ions

Results for all ions, except K^+ and Cl^- indicate little difference in levels between inoculated and control groups and development of infection does not appear to affect levels, even over time (fig 62-65).

Levels of K^+ and Cl^- were quite high (33.2 and 1035.5 mmol/L respectively) for the 2 days post-inoculation with *Bonamia* which were almost double that obtained for the control groups (for K^+,17.8 and Cl^-, 581.0 mmol/L).

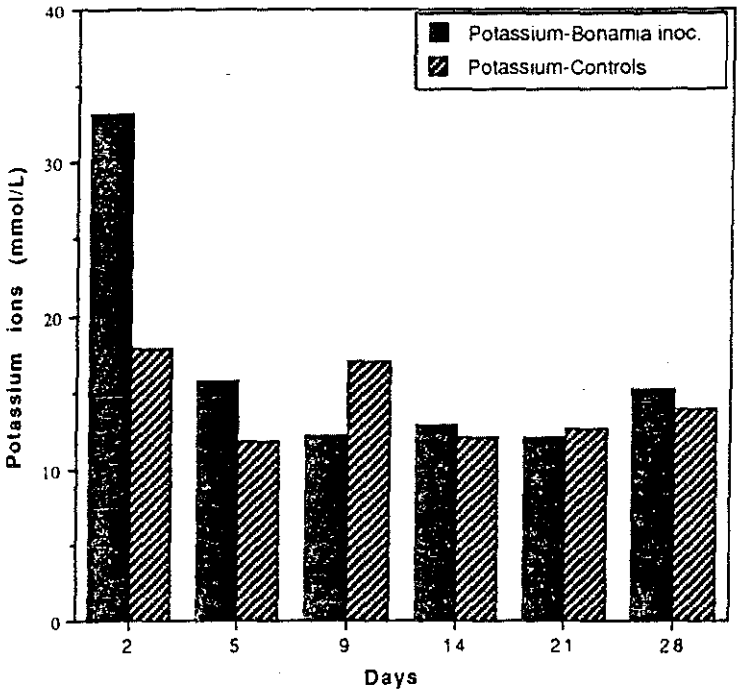


Figure 62. Mean K^+ levels (mmol/L) for *Bonamia* inoculated and saline injected controls over 28 days.

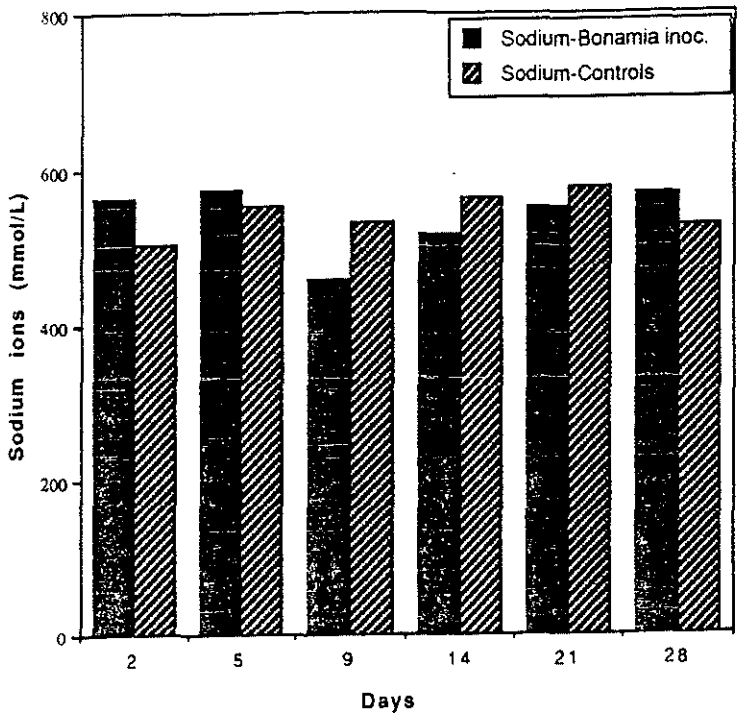


Figure 63. Mean Na^+ levels (mmol/L) for *Bonamia* inoculated and saline injected controls over 28 days.

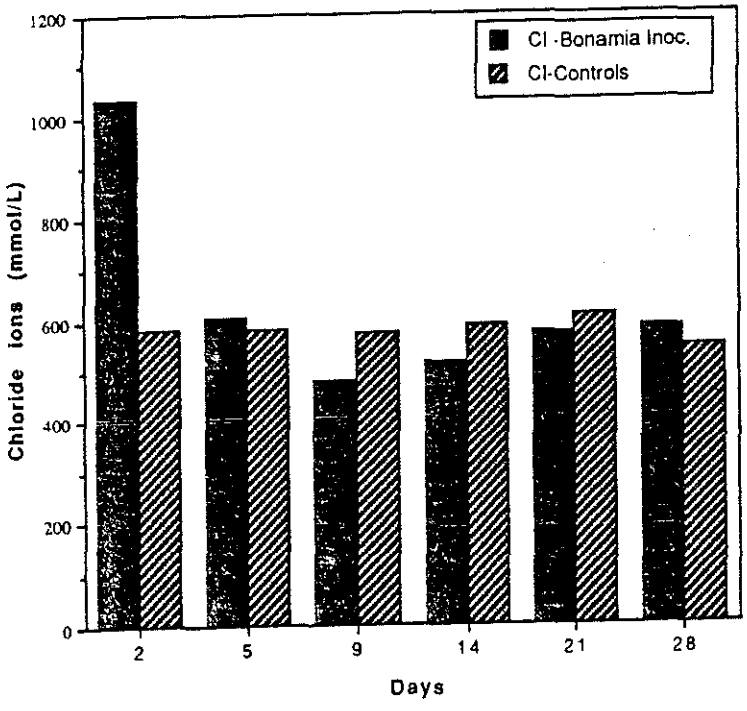


Figure 64. Mean Cl^- levels (mmol/L for *Bonamia* inoculated and saline injected controls over 28 days.

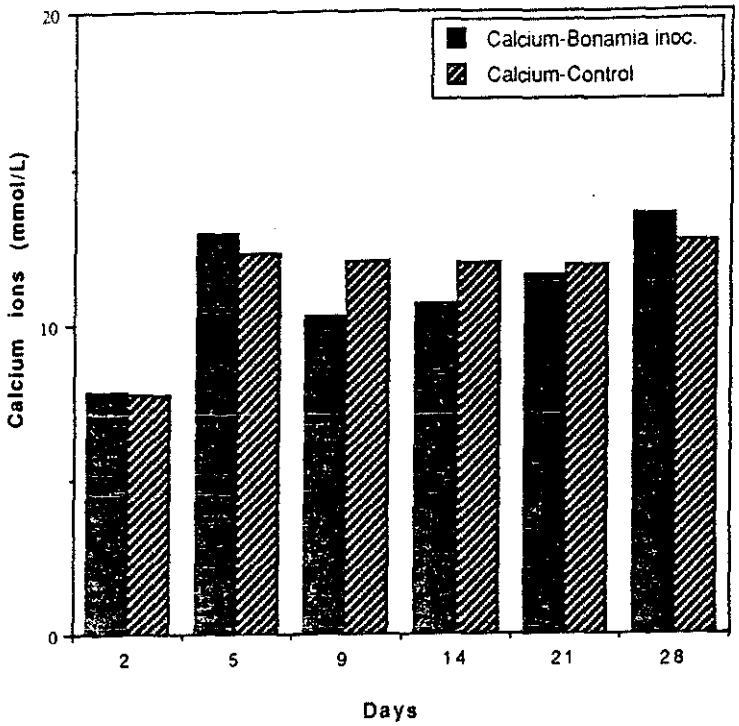


Fig. :- Mean Calcium ion concentration over 28 days

Figure 65. Mean Ca^{2+} levels (mmol/L) for *Bonamia* and saline injected controls over 28 days.

3.1.2 Amylase and Asat

Amylase levels also varied quite considerably both from month to month and between infected and uninfected groups on several months. Highest levels occured in October 1990. Levels may possibly be related to the state of feeding and digestion of the animal (Fig 66). Overall levels varied from 634.9 to 2689.3 1U/L.

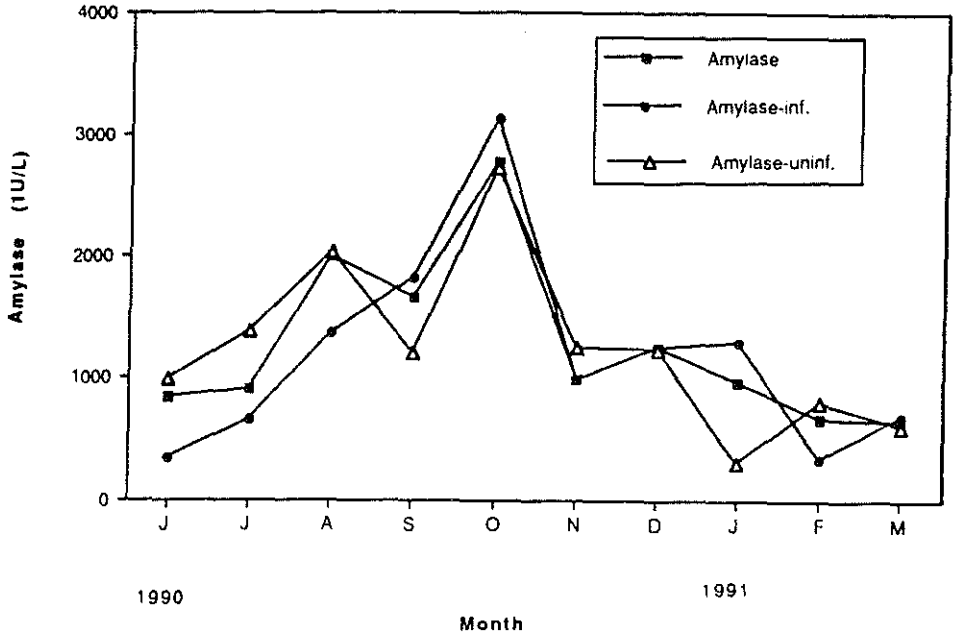


Figure 66. Mean monthly amylase (1U/L) for total sample, infected and unifected oysters from June 1990 - May 1991.

Asat levels examined over a 7 months period were quite variable with no trend in infected or uninfected oysters. Mean levels varied from 0 to 0.5 µKat/L (Fig 67). The highest levels occurred in June 1990

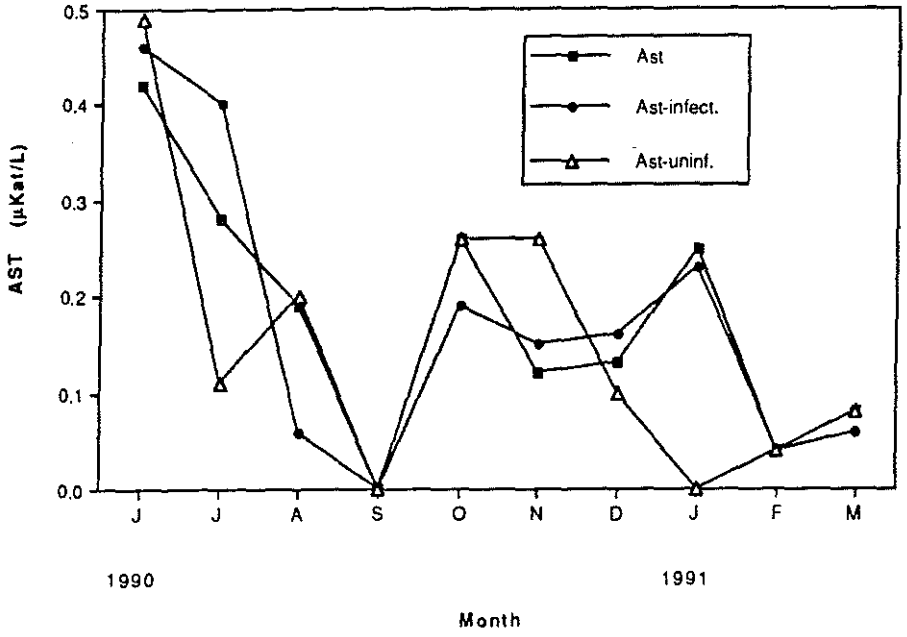


Figure 67. Mean monthly Asat (µKat/L) for total sample, infected and unifected oysters from June 1990 to March 1991.

3.3 Proteins

Protein levels for irish oysters appeared to remain relatively stable from August 1990 to July 1991 varying from 2-3 g/L (Fig 68).

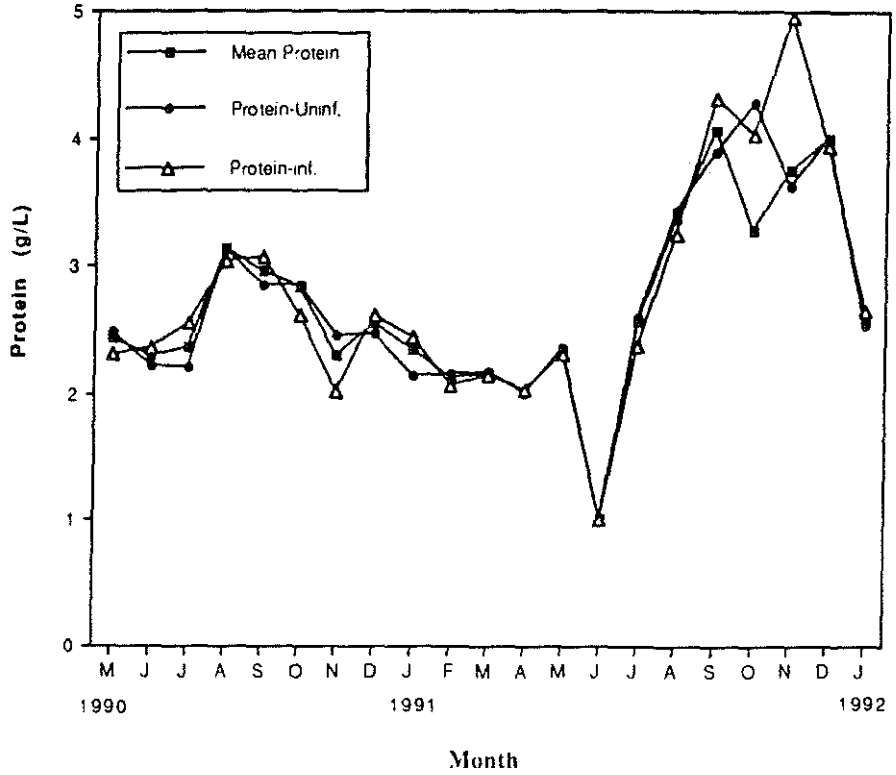


Figure 68. Mean monthly protein levels (g/L) of total sample, infected and unifected oysters from May 1990 - January 1991.

	May'90	June	July	August	September	October	November	December	January '91	February
(a)	2.44	2.30	2.36	3.13	2.96	2.83	2.30	2.55	2.35	2.12
	(0.57)	(0.50)	(0.56)	(0.69)	(0.35)	(0.39)	(0.51)	(0.48)	(0.44)	(0.34)
N=	30	21	19	47	12	49	56	52	48	52
(b)	2.48	2.22	2.20	3.04	3.06	2.61	2.01	2.61	2.43	2.06
	(0.61)	(0.46)	(0.40)	(0.75)	(0.20)	(0.27)	(0.50)	(0.53)	(0.39)	(0.41)
N=	24	9	10	2	6	5	20	24	33	14
(c)	2.31	2.36	2.54	3.13	2.84	2.85	2.45	2.47	2.14	2.15
	(0.32)	(0.54)	(0.68)	(0.69)	(0.47)	(0.39)	(0.41)	(0.41)	(0.49)	(0.32)
N=	6	12	9	45	6	44	36	28	15	38

	March	April	May	June	July	August	September	November	December	January'92
(a)	2.16	2.02	2.35	2.55	3.37	4.06	4.19	3.74	4.00	2.57
	(0.33)	(0.47)	(0.40)	(0.52)	(0.93)	(1.92)	(1.71)	(1.48)	(1.50)	(0.54)
N=	55	56	48	46	49	49	53	52	49	57
(b)	2.14	2.03	2.31	2.38	3.24	4.31	4.03	4.96	3.93	2.64
	(0.44)	(0.53)	(0.45)	(0.43)	(1.13)	(2.55)	(1.66)	(3.26)	(1.59)	(0.55)
N=	16	34	16	9	15	10	19	5	15	20
(c)	2.17	2.00	2.36	2.60	3.42	3.88	4.27	3.61	4.00	2.53
	(0.28)	(0.39)	(0.37)	(0.54)	(0.84)	(1.62)	(1.77)	(1.45)	(1.47)	(0.54)
N=	39	22	32	37	34	39	34	47	34	37

Table 10. - Mean monthly protein levels - SD : Standard deviation (in bracket) of (a) total sample, (b) infected oysters, (c) unifected oysters from May 1990 - January 1992.

However, in August 1991, mean protein concentration rose to 3.37 g/L and peaked in September at 4.06 g/L. This level remained stable until January 1992 when the protein level dropped to 2.57 g/L. A two way ANOVA indicated a significant variation in levels between months (P=0) but not between infected and uninfected oysters (Table 10). Age may be one factor, along with increased feeding to lay down winter stores in explaining increased protein concentrations in the latter part of 1991. Cessation of feeding may be responsible for the reduction in January 1992.

3.2 RELATIONSHIP BETWEEN HAEMOLYMPH COMPONENTS AND PARASITISM, SEASONAL CHANGES, REPRODUCTIVE CYCLE AND GEOGRAPHICAL AREA.

No correlation was found between oyster haemolymph lysozyme and *B. ostreae* infection. Similarly, *B. ostreae* infection did not appear to affect the haemolymph agglutinin title and cells number of the oyster. The lack of correlation between the level of lysozyme, agglutinins title, and the number of cells could be explained by the hypothesis that these factors would not be involved in the defence reaction of the oysters against the parasite.

However, one positive correlation was found between the lysozyme, cells number and the ripe gamets suggesting a possible linkage of these parameters with the reproductive cycle (Table 11), noticely in the elimination of residual gametes after spawning..

	Bomania ostreae	Ripe gametes
Length (mm)	-	0
Flesh weigth (g)	-	+
Proteins (mg/ml)	-	0
Agglutinins title	0	0
Lysozyme (U/ml)	0	+
Cell N (10^6/ml)	0	+

Table 11. - Pearson Correlations. The number of cases was 160 in each sample. (0) no correlation, (+) positive correlation and (-) negative correlation.

On the other hand, as demonstrated by this study, the constituents of haemolymph exhibited a seasonal fluctuation or annual variations in Spain. The highest means in protein, lysozyme, and agglutinin levels were found in summer period and the lowest mean in the winter period. It is possible that the increase in these haemolymph parameters in summer period could have been caused by a high bacterial biomass and also by the increase in food abundance in the same period.(Table 12.

A comparative preliminary study of two geographical areas indicated that the levels of some components of the haemolymph could be influenced by the habitat. We can report higher mean values in all the haemolymph constituents in the exterior than in interior of Ria Vigo (Table 13). The

analysis of variance (ANOVA) showed a significant correlation ($P<0,05$) of these parameters with location and oyster age. However, no significant correlation was found between the location and the *Bonamia ostreae* infection (Table 14).

	Proteins (mg/ml)	Agglutinins titer	Lysozyme (U/ml)	Cells N. (10^6/ml)
Spring	0.57	254.25	69.83	3.51
Summer	0.87	3264.00	132.52	1.98
Autumn	0.58	66.12	9.45	2.77
Winter	0.65	28.18	10.61	2.71

Table 12. - Mean values of the different parameters measured. The number of cases was 40 by season.

	Proteins (mg/ml)	Agglutinin title	Lysozyme (U/ml)	Cells N. (10^6/ml)
Internal	0.54	643.24	42.45	2.24
External	0.80	1163.04	68.76	3.20

Table 13. - Mean values of the different parameters measured. The number of oysters. N=80 by location.

	Proteins (mg/ml)	Agglut. title	Lysozyme (U/ml)	Cells N. (10^6/ml)	*Bomania ostreae*
Age	0.00	0.00	0.00	0.03	0.00
Location	0.00	0.00	0.01	0.00	0.15

Table 14. - Analyses of variance (ANOVA). Differences were considered statistically significant if $P<0.05$ Number of oyster.

These parameters of haemolymph associated with environmental changes must be now studied. Further research, in standard conditions, is required to elucidate the inter-relationships between reproductive cycle, food, temperature, salinity and the bonamiasis.

CHAPTER 4

CONCLUSIONS

Taking into account that mollusc haemolymph is more particularly involved in defence reactions against pathogens and in nutrition, some components were studied in *O. edulis* and compared between infected and healthy oysters to *Bonamia* infection. Their levels were also considered in fonction of the age of oysters, season and rearing location in Spain.

In this study, all parameters, ions and enzyme levels and proteins exhibited large standard deviations. According to Cheng and Rodrick (99), "large standard deviations are characteristic of the haemolymph of certain molluscs with an open circulatory system, the nature of which direct communications of both soluble and paticulate constituents".

Ions leves demonstrated variability over the 28 day period between *Bonamia* inoculated and control oysters. K^+ and Cl^- showed high levels two days post-inoculation compared to those measured in the following days. These high levels may be an initial reaction to the presence of the parasite as it was not observed in control groups.

Mean amylase levels in our study were quite variable from month to month and appeared lower in infected oysters during the first months. Amylase were found in the whole haemolymph and serum of *Crassostrea virginica* (99) but not associated with the haemocytes. C. virginica has a crystalline style and the amylase activity detected in haemolymph may originate from this source.

Aspartate aminotransferase (Asat) and alanine aminotransferase (Alat) appear to be important in maintaining stable amino acid concentrations within the molluscan cell. In these animals, amino acids are continuously lost to the external medium (100) and also may be lost as a result of damage to cells by parasites (101). The levels of Asat in *O. edulis* haemolymph were variable over the experience but no trend appeared with the presence of *B. ostreae*. It has been shown that the presence of *Perkinsus marinus* and *Haplosporidium nelsoni* in their respective hosts resulted in the depletion of free amino acids. So elevated Asat and Alat levels might be an effort on the part of the host to maintain amino acid levels (101;102). The nutritional state of the organism, natural seasonal changes, responses to changes in environmental salinity and effects of pollutants induce also a variablity in the free amino acid pool (102).

Moreover no correlation was demonstrated between the presence of *Bonamia* and the total proteins concentration, agglutinin or lysozyme activity in the present work contrary to other authors. Decreased concentrations of lysozyme (94) and serum protein have been correlated with *Haplosporidium nelsoni* (MSX) systemic infections (96). Finally, it appeared that oyster haemolymph composition displays great variability linked to environmental and seasonal influences as reproductive cycle (42;93).

PART V

OYSTER DEFENCE MECHANISM AGAINST *BONAMIA OSTREAE*

E. BACHERE, V. BOULO, D. HERVIO AND E. MIALHE

PART V

CONTENTS

CHAPTER 1

INTRODUCTION

Both natural infections and controlled experimental inductions of the bonamiasis have shown that, for a same dose of injected parasites, the oysters develop different levels of infections. Beside genetic origin, these differences suggest the involvement of defence reactions in the elimination of the parasite. In order to extend current knowledge of host-pathogen interactions and the processes of elimination or destruction of the parasites, the study of the immune defence reactions, displayed by the oysters, have been undertaken. Defence mechanisms have been investigated at the cellular level, using *in vitro* assays better adapted to analyse the *Bonamia*-haemocytes interactions and to tackle the phenomena of pathogenicity and of induction or inhibition of the haemocyte-mediated reactions, produced by the parasite.

The obligate intracellular nature of *B. ostreae* and its development in the haemocytes have led to a consideration of the process of phagocytosis, the entry and recognition mechanisms and the survival of the protozoan in the phagocytic cells.

The *Bonamia*-haemocyte interactions have been studied using haemocytes of the sensitive species, *Ostrea edulis* which were compared with the haemocytes of *Crassostrea gigas*, which is refractory to the parasite in the field.

Using the knowledge gained from molluscan cell culture (103), studies were undertaken to establish haemocytes primary-cultures of *O. edulis* and *C. gigas*. The availability of purification protocol of the parasites (11), made it possible to develop a model based upon *in vitro* infection of the haemocytes by *B. ostreae* (104). In this method, haemocyte primary-cultures were prepared on slides. After adhering and spreading of the haemocytes, a suspension of purified parasites was added. It has been shown that, after 30 minutes of incubation, the parasites are internalized in the haemocytes of the two oyster species, *O. edulis* and *C. gigas*. The three haemocytic types, optically identified as granulocytes, small hyalinocytes and large hyalinocytes, were parasitized. However, the infection rates are higher for granulocytes than for the large hyalinocytes, the difference being more pronounced in *C. gigas* than in *O. edulis*. Such a *in vitro* system facilitated the study of the entry mechanisms (105; Annexed). Based on ultrastructural features and on Cytochalasin-B treatment of the haemocytes or the parasites, it has been demonstrated that *B. ostreae* enters in the haemocytes of the two oyster species by host-specified phagocytosis.

Taking into account these results, the study of phagocytosis processes was undertaken, focusing on recognition mechanisms and on the intracellular evolution of *B. ostreae* in the haemocytes of *O. edulis* and *C. gigas*. However, although the use of haemocyte monolayer primary-culture permitted quantitative analyses of infection rates, it was observed that infected haemocytes tend to come off the slides and so were lost during the fixation and coloration steps. In this way, the protocol for infection of haemocyte primary cultures were improved by using primary-cultures of haemocytes in suspension.

Oxidative microbicidal activity linked to the phagocytosis and known as "Respiratory Burst", has been investigated in the two oyster species using chemiluminescence. The phenomenon of chemiluminescence is commonly

used in vertebrates (106;107) and gastropods (108) and corresponds to the production by the haemocytes of oxygen metabolites in response to particulate stimulation. The potential role of this oxygen dependent mechanism has been studied with respect to phagocytosed *B. ostreae*, because of the mechanisms general microbicidal action.

CHAPTER 2

MATERIALS AND METHODS

2.1. HAEMOCYTE PRIMARY-CULTURE INFECTIONS

2.1.1. Preparation of haemocyte primary-cultures

The haemolymph samples were collected from the pericardial cavity of the oysters, *O. edulis* and *C. gigas*, and simultaneously 2-fold diluted with anti-aggregant Alsever Solution (AS) (109). Haemocytes counts were performed using a Malassez hemacytometer and 2 x 10^5 haemocytes were sampled in Eppendorf tubes. After centrifugation, the AS/haemolymph mixture was removed. Then the cell pellets were resuspended in 2 ml of Filtered Sea Water (FSW) in order to obtain a AS final concentration below 3%. At this concentration of AS, the suspended haemocytes recover their activity and phagocytic capacity.

Purified parasites could be added to the haemocyte primary culture at a parasite to haemocyte ratio of 5 to 1.

After the phagocytosis assay, the cell suspensions were cytocentrifuged, fixed and colored for histological analysis and estimation of the infection rates for 100 haemocytes.

This protocol has been used for the different experimentations consecutively carried out.

2.1.2. Recognition mechanisms

- Involvement of *B. ostreae* epitopes in recognition

The purified parasites were incubated one hour in a solution of specific monoclonal antibodies (MAB), 20B2 or 15C2, or in Filter Sea Water (FSW). After washing, they were added to the haemocyte suspensions of *O. edulis* and *C. gigas*. After two hours of incubation, the haemocyte suspensions were cytocentrifuged, fixed and stained. The infection rates were evaluated by histological examination of 100 haemocytes.

- Opsonic effect of oyster haemolymph components

Haemocyte primary-cultures in suspension were prepared as described above. In other respect, *O. edulis* and *C. gigas* haemolymphs were collected and centrifuged to obtain serum samples. Then, purified parasites were incubated one hour at 18°C in the different serum samples and FSW as control. After that, the parasites were added to the haemocyte suspensions of the two oyster species for 2 hours at 18°C. The infected primary-cultures were cytocentrifuged, fixed and stained. The infection rates were determined by histological examination of the cells.

2.1.3. Intracellular evolution of *B. ostreae* in the haemocytes

Primary cultures of haemocytes in suspension were infected with purified *B. ostreae* and kept at 18°C with antibiotics (Vancomycin, 50 µg/ml; Bacitracin, 150 µg/ml; Penicillin, 150µg/ml). At times 12h., 24h., 48h., 72h., 96h., 120h., and 144h, the suspensions were cytocentrifuged and observed. The infection rates were determined from the observation of 100 haemocytes by preparation.

2.2. CHEMILUMINESCENCE

2.2.1. Chemiluminescence assay (9)

Haemolymph samples were collected from the pericardial cavity of the oysters and simulteanously 2-fold diluted with Alsever Solution. The haemolymph samples were used either separately or pooled (10 individual haemolymphs).

The haemocytes were immediately counted and sampled as previously described in paragraph 2.1.1.

The generation of chemiluminescence (CL) was measured with a liquid scintillation counter (Tri-Carb 2200 CA, Packard) in the out-of-coincidence mode. The counter was set on repeated cycles and sequential 0.5 min counting periods at fixed intervals, generally for 5 h. The assays were performed at room temperature. Each plastic scintillation vial contained 2 x 10^5 haemocytes. "Luminol" (Sigma) solution (10^{-1}M) in DMSO (dimethyl sulfoxide, Sigma) was introduced in each vials at a final concentration of 10^{-4}M in a total volume of 2 ml of Filter Sea Water (FSW). The CL base activity of the haemocytes was recorded for a first run. Then the stimulant, Zymosan or *B. ostreae*, was added at a selected particle-to-haemocyte ratio and the CL responses were measured.

Zymosan. Zymosan particles (Sigma) were suspended in FSW (40 mg/ml^{-1}), heated for 30 min at 100°C, washed, suspended in FSW, dispensed and stored at -20°C. In the CL assays, a particle-to-haemocyte ratio of 80/1 was used.

B. ostreae. The preparation of the parasites for CL assays is given in paragraph 2.2.3.

2.2.2. Individual chemiluminescence activity

Individual haemolymph samples were prepared for *C. gigas* (3 assays with respectively, 9, 11 and 18 animals) and for two strains of *O. edulis*: oysters from Mediterranean, a *Bonamia*-free breeding area and Quiberon F1 oysters. The latter strain was composed of oysters originating from spat produced in hatchery from old oysters collected from the chronically infected area of Quiberon Bay and were considered as possibly "resistant".

In parallel with chemiluminescence assays, the different haemocyte samples were cytocentrifuged, fixed and stained for histological determination of the different cell populations. For each sample, 100 haemocytes were examined and classified into granulocytes and hyalinocytes, small, medium and large, on the basis of optical morphological criteria.

The number of haemocytes per ml of haemolymph were also counted for the different samples.

2.2.3. Chemiluminescence activity against *B. ostreae*

First assay:

Chemiluminescence assays were carried out as previously described (2.2.1), using zymosan as control of the stimulation and purified parasites, live or heat killed (100°C) at a ratio of 40 parasites per haemocyte.

Second assay:

Different parasite suspensions were added to the haemocyte samples at a ratio of 40 per haemocyte:

- live *B. ostreae*
- live parasites treated with L-Tartrate (10^{-2}M, 30 min), an inhibitor of some acid phosphatases (111)
- heat-killed parasites
- parasites sonicated in order to release the acid phosphatase which is localized in the dense body as previously shown using ultracytochemical method (118).

The following samples were prepared as controls:

- non stimulated haemocytes
- zymosan stimulated haemocytes (80/1)
- haemocytes stimulated with L-Tartrate-treated zymosan

Third assay:

Haemocyte samples were inoculated with purified parasites, either alive either heat killed. After one hour incubation, necessary for the internalization of the parasites, the haemocytes were used in CL assay and stimulated with zymosan as previously described (2.2.1)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. RECOGNITION MECHANISMS

3.1.1. Involvement of *B. ostreae* membrane epitopes in recognition.

In some protozoan, parasites of vertebrate macrophages the recognition processes often involve membranous components which can be identified by monoclonal antibodies (112). The availability of *B. ostreae* specific monoclonal antibodies (5) made it possible to check parasite membranous epitopes involved in recognition mechanisms using parasites incubated with 20B2 or 15C2 MAB.

The results shown in figure 69 indicate no significant difference between the phagocytosis of treated and untreated *Bonamia* (P= 0.503, 0.05%) in *O. edulis* haemocytes. For *C. gigas*, the treatment of the parasite with the 20B2 monoclonal led to an significant increase of the infection rate, 36% instead of 26% for untreated *B. ostreae* suggesting a opsonizing effect of the antibody. However, the slight reduction of the infection rate linked to the 15C2 antibody treatment was not significantly different from the control (Fig 69).

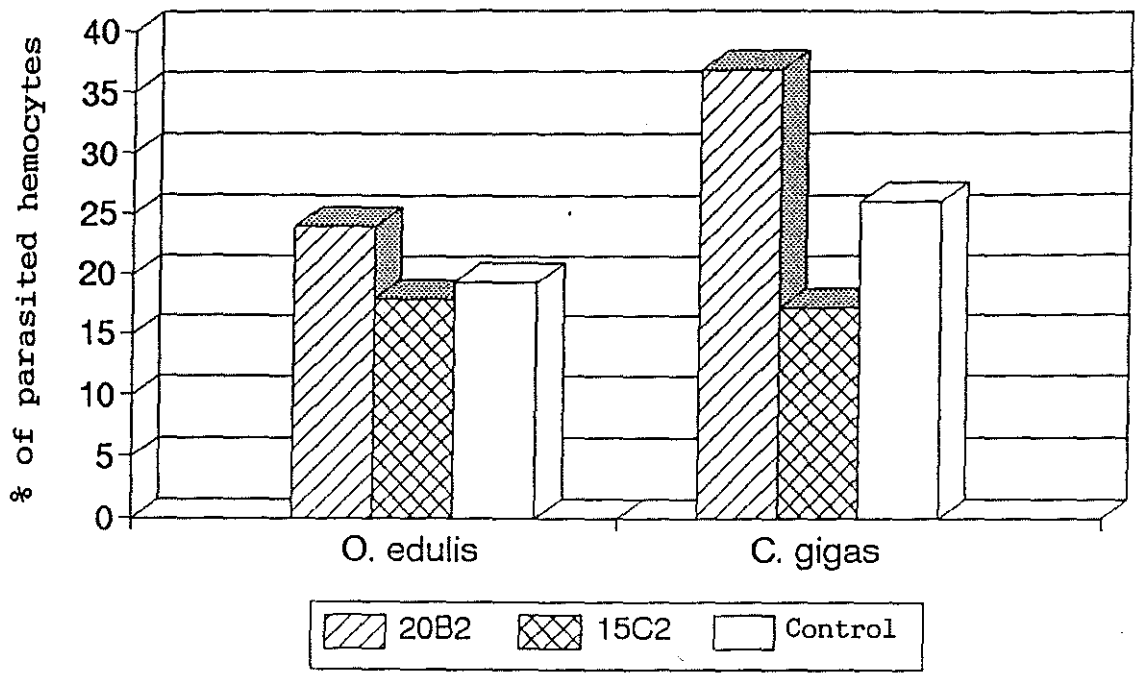
3.1.2. Analysis of opsonic effect of haemolymph of *O. edulis* and *C. gigas*.

Lectins are glycoproteins which bind to foreign particles present in the haemolymph and which promote the phagocytosis. Numerous studies have been carried out on gastropods, showing the involvement of lectins in recognition and phagocytosis phenomenons (113;114).

In order to investigate if such molecules occur in the phagocytosis of *B. ostreae* by *O. edulis* and *C. gigas* haemocytes, the infection rates have been compared when the parasites was previously incubated or not in the serum of *O. edulis* or *C. gigas*. The infection rates determined by histological examination of cytospin are presented in table 15.

For *O. edulis*, the previous incubation of the parasites in the serum of the two species, led to a non significative reduction of the infection rates (t=-0.762, ddl=9, 0.05%). For *C. gigas*, a significative increase of the infection rates was observed when the parasite was incubated in *O. edulis* serum. On the other hand, the increase of the infection rates from 27% to 37.3% observed when the parasites was incubated in *C. gigas* serum, was non significative (t=2.238, ddl= 5 for 0.005%; for 10% this increase was significative)(Fig 70).

These results would show an opsonising effect of the *O. edulis* serum and, in less degree, of *C. gigas* serum, on the phagocytosis of *B. ostreae* by *C. gigas* haemocytes. Concerning the haemocytes of the host species *O. edulis*, an opsonising effect of the serum could not be demonstrated in the recognition and phagocytosis of the parasite.



Animals treatment		1	2	3	4	5	% Infection (moy. ± SD)
20B2	O.e.	26	32	25	21	16	24 ± 5.9
	C.g.	38	22	35	35	54	36 ± 11.4
15C2	O.e.	17	21	33	4	15	18 ± 10.5
	C.g.	22	15	10	7	32	17.2 ± 10
FSW	O.e.	21	6	36	12	22	19.4 ± 11.4
	C.g.	33	12	30.9	27	27	26 ± 8

Infection rates obtained for 5 animals of the species, *O. edulis* (O.e.) and *C. gigas* (C.g.).

Figure 69: Effect of *B. ostreae* treatment with specific monoclonal antibodies, 20B2 and 15C2, on the recognition haemocyte-parasite. Control: *B. ostreae* incubated in filtered sea water

Hémocytes	B.o. + Sérum	Samples tested										Control Bo + FSW
		1	2	3	4	5	6	7	8	9	10	
O.e. O.e.	O.e. C.g.	65 34	26 31	21 36	32 37	29 19	24 35	25 17	39 36	37 22	41 45	37
C.g. C.g.	C.g. O.e.	46 44	21 42	46 54	38 32	26 37	47 31					27

Middle percentage of infection rates recorded

O.e. x O.e.: x = 34% ± 12.8%

O.e. x C.g.: x = 31% ± 9%

C.g. x C.g.: x = 37.3% ± 11%

C.g. x O.e.: x = 40% ± 8.6%

Table 15: Individual analysis of the opsonising role of the haemolymph in the phagocytosis of *B. ostreae* in *O. edulis* and *C. gigas*.

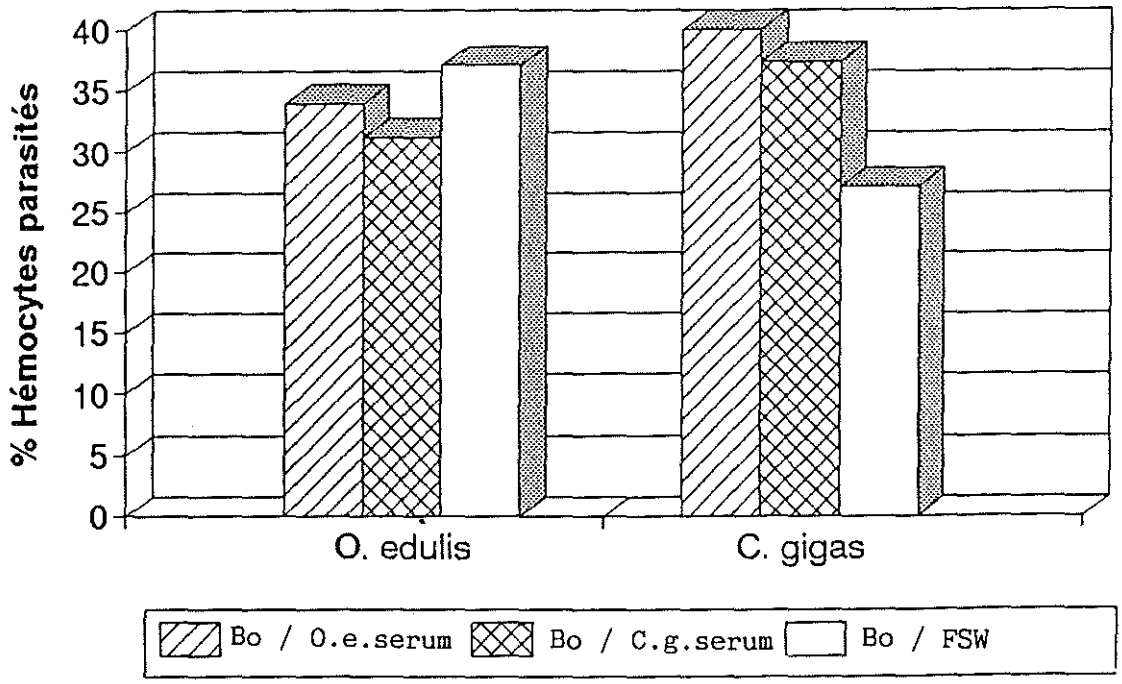


Figure 70: Opsonising effect of the haemolymph on the phagocytosis of *B. ostreae*, comparison of the middle percentages of infected haemocytes.

3.2. Analysis of the intracellular evolution of *B. ostreae* in the haemocytes of *O. edulis* and *C. gigas*.

The similarity of the early stages of *in vitro* interactions between *B. ostreae* and the haemocytes of the two oyster species, led to a consideration of the intracellular development of the parasite. It was hypothesied that *B. ostreae* developed in the haemocytes of its host, whilst it would be eventually destroyed in *C. gigas* haemocytes.

We must point out that, in these assays, the parasites were not eliminated from the medium after one or two hour incubation.

As shown in table 16, at 12h, the middle infection rates were not different between the two oyster species (29% for *O. edulis* and 37% for *C. gigas*) and approximately comparable to those obtained after 24h incubation. However, as early as 24h, haemocytes displayed symptoms of impaired cellular function. After 4 days, the cells were severely damaged. The parasites however appeared in good condition. The infection rate did not increase, suggesting that the phagocytosis activity of the haemocytes stopped early in the incubation. So, neither multiplication in the *O. edulis* haemocytes, nor degradation in the *C. gigas* haemocytes were demonstrated.

Animals / Time	1	2	3	4	5	6	7	8	X	Species *O. edulis* (OE) ou *C. gigas* (CG)
12 h	38	40	34	25	7	9	20	25	29	OE
	52	57	65	17	35	30	14	28	37	CG
24 h	74	56	60	47	19	22	33	52	45	OE
	nd	30	30	32	34	47	28	56	36	CG
48 h	61	nd	42	22	28	23	20	33	32	OE
	11	19	36	22	20	43	42	36	28	CG
72 h	63	21	59	35	23	12	33	33	34	OE
	15	42	37	42	35	nd	33	49	36	CG

Table 16: Variation of the phagocytosis rates of the haemocytes for *O. edulis* (OE) and *C. gigas* (CG) (%) as a function of time.
nd: non determined

3.3. OXIDATIVE MICROBICIDAL PROCESSES OF THE HAEMOCYTES OF *O. EDULIS* AND *C. GIGAS* RELATED TO THE PHAGOCYTOSIS OF ZYMOSAN PARTICLES AND *B. OSTREAE*.

3.3.1. Analysis of individual variability of the chemiluminescence activity

The chemiluminescence response of individual oysters were analysed and showed great interspecific and also intraspecific variability (91). So,

we tried to look for a possible correlation between the chemiluminescence response and the haemogram. The percentages of the different cell types, the CL activities and the total number of haemocytes per samples are presented in table 17.

No correlation could be found between the haemograms and the chemiluminescence responses of the corresponding haemocyte samples in neither *C. gigas* or *O. edulis*. However, it appeared during the observations that the optical microscopic identification of haemocyte types on the basis of morphological features was difficult and were unreliable.

	GRANULOCYTES (%)	HYALINOCYTES (%) LARGE	MEDIUM	SMALL	BLOOD COUNT (cells/ml)	CHEMILUMINESCENCE (cpm)
C. gigas						
- Exp. A (n= 9)	19.4 ± 9.9	10.3 ± 9.4	16.3 ± 7.1	53.8 ± 19.8	$7.9.10^5$ ± $5.9\ 10^5$	$28.0.10^4$ ± $23.0.10^4$
- Exp. B (n=11)	29.2 ± 15.3	16.9 ± 9.7	26.4 ± 12.7	27.3 ± 18.6	$2.8.10^5$ ± $0.9\ 10^5$	$65.6.10^4$ ± $42.3\ 10^4$
- Exp. C (n=18)	39.6 ± 12.4	34.5 ± 13.1	15.5 ± 7.4	10.2 ± 7.6	$4.6.10^5$ ± $2.6\ 10^5$	$24.7.10^4$ ± $17.3\ 10^4$
O. edulis						
Méditerranée (n= 5)	42.6 ± 19.8	27.8 ± 15.0	16.8 ± 4.3	12.8 ± 10.0	$5.7.10^5$ ± $2.9\ 10^5$	$41.4.10^4$ ± $33.5\ 10^4$
Quiberon F_1 (n=9)	41.3 ± 15.0	23.2 ± 7.8	29.8 ± 14.1	5.5 ± 5.0	$25.1.10^5$ ± $17.0\ 10^5$	$63.7.10^4$ ± $31.4\ 10^4$

Table 17. Haemocytic numeration and chemiluminescence in *C. gigas* and *O. edulis*.

In the F1 Quiberon oysters the chemiluminescence activity of the haemocyte sample ranged in values observed for any one oyster strain or species. However, the number of haemocytes counted were much higher than the number generally observed. The F1 Quiberon oysters presented haemocyte numbers between $8x10^6$ and $42x10^6$ per ml of haemolymph when generally flat oysters display about $3x10^6$ to 10^7 haemocytes/ml.

3.3.2. Role of the oxidative metabolism of the haemocytes of *O. edulis* and *C. gigas* against phagocytosed *B. ostreae*

It has been shown that the internalisation of *B. ostreae* in the haemocytes of the two species is carried out by phagocytosis. It has also been shown that the phagocytosis of zymosan triggers the production of oxygen metabolites and chemiluminescence activity. The oxygen intermediates found in vertebrate phagocytes are known to be as powerfull microcidal products effective against a variety microorganisms and parasites (115;116;117).

Several *in vitro* experimentations were carried out to analyse the interaction between *B. ostreae* and the oxygen dependent cytotoxic mechanism of *O. edulis* and *C. gigas* haemocytes.

First assay:. The figure 71 shows chemiluminescence activity peak values obtained during the phagocytosis of zymosan, alive or killed *Bonamia* by the haemocytes of the two oyster species.

When the haemocytes stimulated by zymosan developed chemiluminescence activity, no response was recorded for the haemocytes with *Bonamia* in either oyster species. However, the cytocentrifuged samples showed that the parasites were internalized.

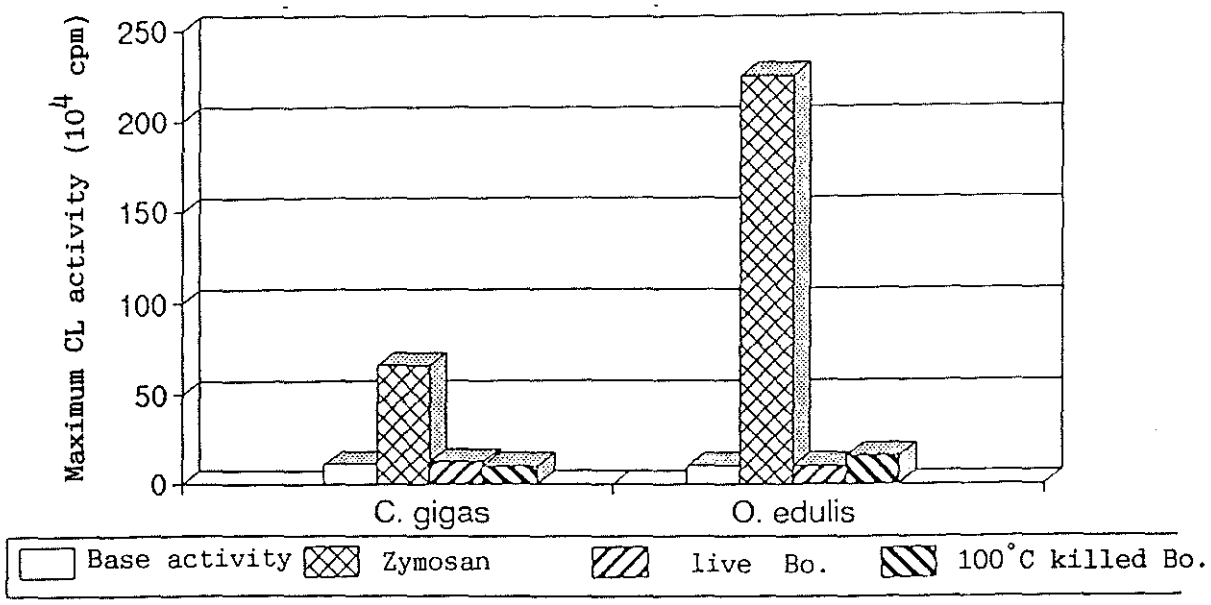


Figure 71: Chemiluminescence activity of *O. edulis* and *C. gigas* haemocytes stimulated by zymosan, live or heat-killed *Bonamia*
80 zymosan particles/1 haemocyte
40 parasites/1 haemocyte

Second assay: Taking into account these results and on the basis of knowledge on vertebrate protozoan such as *Leishmania* an investigation was made of the role of acid phosphatase in *Bonamia*. Indeed, it was possible to localize and characterize an acid phosphatase activity in the dense body of *B. ostreae* (118). Such enzymes in Leishmania membranes are involved in the inhibition of the phagocyte capability to produce superoxide ions and oxygen intermediates (111). So, several experiments were carried out to investigate if the acid phosphatase was involve in the lack of chemiluminescent response by oyster haemocytes, by treating the parasites with inhibitors or heating.

Whatever the oyster species, the addition of any kind of the parasite samples did not generate chemilumunescence activity of the haemocytes when compared to the controls.

Third assay: A study was made of the possible interference of *B. ostreae* with haemocyte capability to generate oxygen metabolites by stimulating haemocytes infected by *Bonamia*.

The chemiluminescence activity recorded for the different samples of

O. edulis and *C. gigas*, were not different from the control, zymosan stimulated haemocytes (Fig 72). This experiment did not shown any inhibitory effect by *B. ostreae* of zymosan induced chemiluminescence activity. On the other hand, the results suggest that the internalisation of *B. ostreae* in the haemocytes of the two oyster species do not interfere with the subsequent zymosan stimulation of the haemocyte "Respiratory Burst" and the triggering of the oxidative metabolism.

We can conclude that the phagocytosis of *B. ostreae* would not trigger the oxidative metabolism of the haemocyte either for the host species *O. edulis*, or the refractory species *C. gigas*.

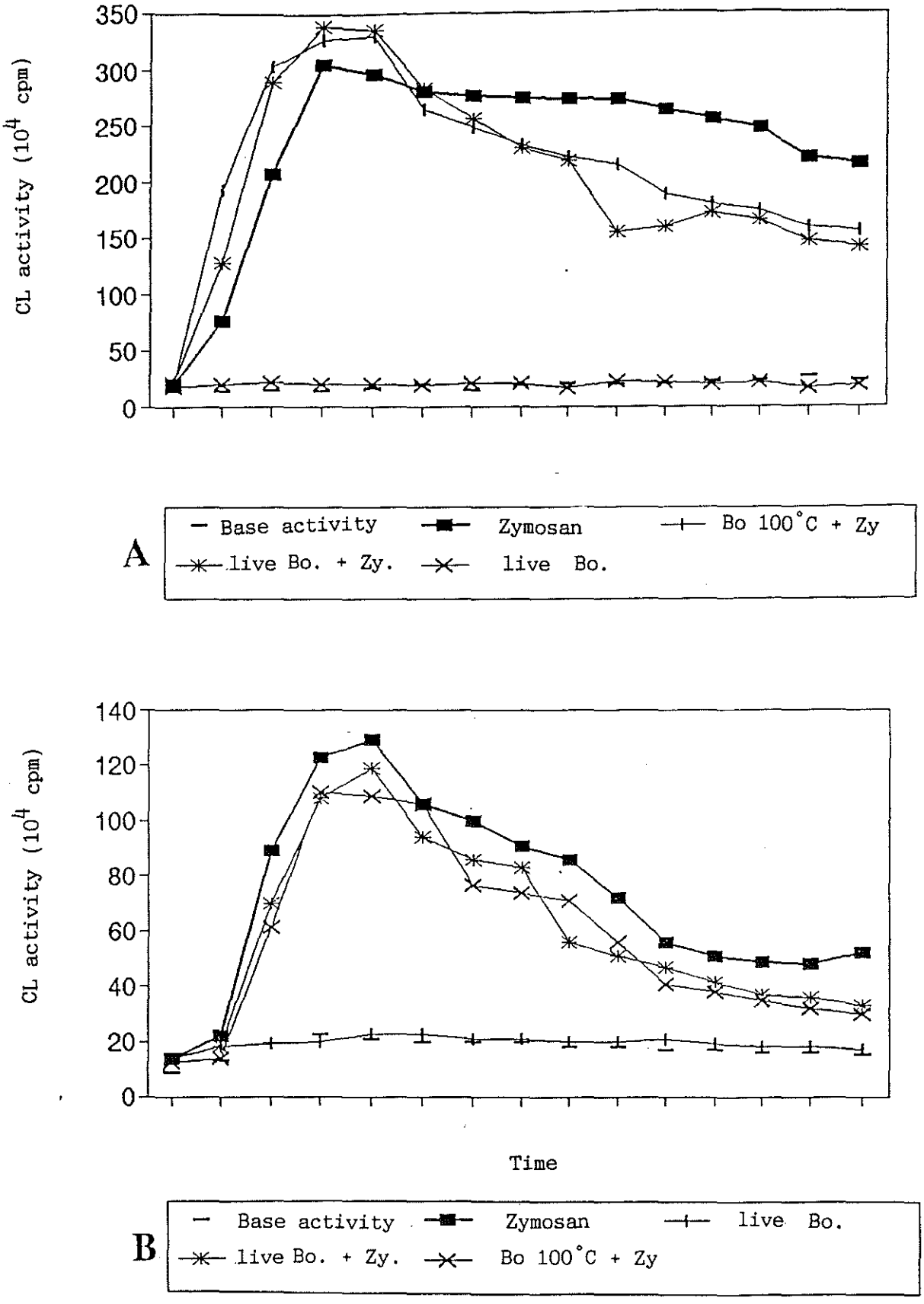


Figure 72: Effect of phagocytosed *B. ostreae*, live or heat killed, on the further chemiluminescence activity of the haemocytes stimulated with zymosan for *O. edulis* (A) and *C. gigas* (B).

CHAPTER 4

CONCLUSIONS

The main objective of this work was the investigation of haemocyte-*Bonamia* interactions since phagocytosis is an essential feature of molluscan immunity and especially in view of the susceptibility of *O. edulis* to *Bonamia* and the refractoriness of *C. gigas* to this pathogen.

The availability of purified parasites and a technique for haemocyte primary culture made it possible to establish a protocol for *in vitro* infection, suitable for the examination of host-pathogen.

For the two oyster species, the parasite enters all cell types by phagocytosis as shown by its sensitivity to Cytochalasin B. The phagocytosis, which is the main entry mechanism of the protozoan parasites, depends on receptor-ligand interactions occuring at the plasma membrane level between the host cell and the parasite. So, with the availability of *Bonamia* specific monoclonal antibodies, some masking assays of the parasite epitopes were carried out. For *O. edulis*, the absence of effect on the phagocytosis rate would indicate that the epitopes masked by the 20B2 and 15C2 monoclonal antibodies are not involved in recognition processes and the phagocytosis of the parasite. However, in some protozoans, parasiting vertebrates, such as Trypanosomas, Plasmodiums or Leishmanias, the masking of membrane components by specific antibodies lead to an inhibition of the entry of the parasites in their host cell (112;119;120). However in *O. edulis*, the treatment of *B. ostreae* with the 20B2 monocloal antibody leads to an increase of the phagocytosis of the parasite. This opsonizing phenomenon could be related to a non-specific binding of antibodies often observed on *C. gigas* haemocytes and suggests the presence of immunoglobulin receptors on the surface of some cells.

The opsonizing effect of the haemolymph was also investigated. In *C. gigas*, the serum of *O edulis* and *C. gigas* increases the phagocytosis of the parasite, whereas in *O. edulis* haemocytes no effects were observed. The phenomenon of opsonization by the haemolymph has been demonstrated and lectins have been identified mainly in gastropods (113;114). It may be assumed that the haemocytes of *C. gigas* have higher affinity in terms of receptors than *O. edulis*. Also, the phagocytosis of *B. ostreae* by the haemocytes of *O. edulis* would not require the involvement of lectins as recognition and opsonizing factors.

The post-phagocytic events were investigated because of the similarity of the early infection events between the haemocytes of *O. edulis* and *C. gigas*. However, because of morphological and functional impairment of the haemocytes *in vitro* maintained for more than a few hours, the study of the intracellular evolution of the parasite was unsuccessful. These results clearly show the limits of *in vitro* culture systems in molluscs and the need for further research into cell cultures and the formulation of culture media adapted to molluscan haemocytes.

The haemocyte-mediated cytotoxic mechanisms were studied for *O. edulis* and *C. gigas*, firstly with respect to oxygen-dependent activity related to phagocytosis. The technique of chemiluminescence was adapted to bivalve haemocytes and now can be used to analyse large numbers samples. In

vertebrates, the chemiluminescence is thought to provide good estimation of the phagocytic capacity of an animal (121). Indeed, using chemiluminescence assays, it has been possible to assess (check) the effect of some pollutants and antibiotics used in aquaculture, on the phagocytosis capacity of *C. gigas* haemocytes (122). The logical development of the research would be the investigation and explanation of the great variability in the chemiluminescent responses. It would also seen highly desirable that the cell lineages and precise functions of the various haemocyte types should be properly established. This work would require the development of additional techniques for the identification and characterisation of cell types and functions. To this end the URPIGM (IFREMER) have already undertaken the preparation of specific monoclonal antibodies against the haemocytes of *C. gigas*. Such reagents would provide antigenic cell typing and lineages. The analysis and characterization of the monoclonal antibodies are now in progress.

Preliminary results show that only the hyaline cells display oxygen dependent mechanism related to phagocytosis when the granular phagocytes are devoid of this killing system. The role of the molluscan granulocytes remains enigmatic.

Thus, it would be very important to investigate if this characteristic could be really related to a difference of sensitivity to *Bonamia*.

Finally the interaction of the oxidative microbicidal processes was investigated with respect to *B. ostreae*, leading to the evidence that the phagocytosis of the parasite by the haemocytes of the two oyster species was not accompanied by chemiluminescent activity. These *in vitro* experiments strongly suggest that *B. ostreae* is able to avoid or inhibit the microbicidal processes of the host cells. Such adaptations to avoid oxidative killing system are known in vertebrate parasites, mycobacteria (123), trypanosomas (124) and leishmanias (125). However, the involvement of cytokines might also be looked for in bivalves, since in vertebrates these molecules activate the killing mechanisms of the immune cells.

Finally, in order to explain the refractory nature of C. *gigas* and the processes of the elimination of *B. ostreae* in this oyster, other microbicidal systems might be investigated in oysters, such as lysosomal enzymatic activities or L-arginine dependent system which has recently recently been shown to be efficacious against trypanosomes (126).

PART VI

ASSESSMENT OF RELATIVE OYSTER STRAIN RESISTANCE TO BONAMIA OSTREAE

E. BACHERE, V. BOULO, N. COCHENNEC, S. CULLOTY, D. HERVIO, E. MIALHE AND M. MULCAHY.

CONTENT

CHAPTER 1

INTRODUCTION

The endemic feature of the bonamiasis had led to severe reductions in the production of flat oyster *O. edulis* throughout european countries over the last decade. Replanting of cleaned areas results in the reappearance of the disease (127). How the parasite maintains itself in infected areas has not yet been elucidated and methods to remove it permanently have not yet been discovered.

In an attempt to maintain culture of flat oyster, farming techniques might be modified to limit the prevalence and mortalities: reduction of stocking densities and culturing at different depths, limiting manipulations of oysters. However, the effects of environmental parameters cannot be controled. The introduction of alternative species of *Ostrea* has been unsuccessful (128). So, oyster farmers, particularly in France have turned to the production of *Crassostrea gigas* (129).

A long term approach to the problem of bonamiasis has to be adopted. Beside prophylaxis based on diagnosis, the survival of flat oyster productions depends on the selection of resistant strains which might be the most effective solution. Two ways for selection can be developed:

- a natural selection linked to the identification of surviving or less sensitive animals taken in the field. This process takes several generations. Field trials were carried out in Ireland to examine the relative susceptibility of Irish oyster populations to *Bonamia* and to determine if oysters bred from survivors of bonamiasis showed any resistance to the parasite.

- the production of resistant animals by exerting selection pressure on successive lineages. This last strategy depends on the availability and reliability of techniques of experimental pathology. The parameters for the reproduction of the disease have been established.

CHAPTER 2

MATERIAL AND METHODS

2.1 FIELD TRIALS

Three field trials were carried out to test the resistance of Cork F1 oysters bred from survivors of the disease. Bonamiasis has been recorded in Cork Harbour since 1986, and the Cork oysters are the progeny of survivors. Disease free oysters were obtained from Tralee Bay, Galway Bay and Belmullet, all situated on the west coast of Ireland.

Cork survivors, approximately 1000 broodstock of 2-4 years of age from the North Channel, were conditionned and spawned in the spatting ponds on Brick Island in 1987 and 1988. When the larvae reached 255 µm, they were settled on mussel shell. The spat were then laid out on beds.

2.1.1 Trial 1:

In January 1990, batches of 140, 145 and 164 three year old oysters, from Cork, Galway and Belmullet, were placed in bags on trestles in the North Channel of Cork Harbour. These trestles are exposed at low tide for several hours.
The prevalence of *Bonamia* were calculated on initial sampling and subsequently, in March, June and October. The number of empty shells was counted and removed and the % cumulative mortality (% CM) estimated using the following formula:

$$\% \text{ CM} = \frac{(\text{Sample size at To} - \text{living sample size at Tx}) \times 100}{\text{sample size at To}}$$

2.1.2 Trial 2:

Trial 2 was the same as Trial 1 except that oysters were obtained from Tralee instead of Belmullet. In April 1991, 126, 280 and 404 oysters from Cork, Galway and Tralee respectively were placed in bags on trestles. Samples were taken in April prior to initial relaying and subsequently in June and October.

2.1.3 Trial 3:

This trial was begun in November 1991, again using oysters from Cork, Galway and Tralee. Initial samples was taken and subsequently in February and May 1992.

2.2 EXPERIMENTAL INFECTION - LABORATORY TRIALS

2.2.1 Establishment of the infection protocol

Oysters: Three years old flat oysters *O.edulis* were collected from some places of Etang de Thau (Mediterranean) where *B.ostreae* is not found. Other three and four-year-old flat oysters were collected from Quiberon Bay and from the Penzé River which are both infected areas.

Parasites: *B. ostreae* was purified according to the protocol of Mialhe *et al* (11). Briefly, highly infected oysters were selected by

examination of heart smears using light microscopy. After homogenisation 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 filtered sea water (0.22µm). The parasite viability was assessed according to the test of Parks *et al.* (130). *B. ostreae* cells were counted using a Malassez-cell haemocytometer .

Experimental infections: A portion of the right hand oyster valve was removed on a dorso-ventral axis, taking care to leave the gills and the mantle intact.
The parasite suspensions, or filtered sea water (0.22µm) for controls, were inoculated (100µl) into the connective tissues of the digestive gland using a Terumo syringe (1ml) equipped with a G26 1/2" needle. After injection, the oysters were kept emerged for half an hour before re-immersing in sea-water.

Oyster maintenance: According to the size of the samples, which consisted of 30 to 100 oysters, fifty-liter or two-hundred-liter tanks were used. The filtered sea-water (10µm) was kept at ambient temperature (approximately 18°C) and changed weekly. The oysters were fed and the mortalities monitored daily.

Detection of B.ostreae: Heart smears of dead and sacrificed oysters were used to detect the parasites. Where the parasite detection had to be performed without killing the oysters, a gill biopsy was used for preparing smears. In either case, the tissue pieces were dried on blotting paper. The smears were then air-dried, fixed with methanol and stained with a Hemacolor kit (Merck) by quick immersion (30 sec) in each reagent. These histological preparations were examined at x1,000 magnification under a light microscope.
The infection level was estimated as follows:

- negative results, referred to (-), were recorded for samples for which no parasite was detected after 5 minutes examination.
- low positive infections, referred to (+), were recorded when about ten parasites were observed during the same time.
- medium positive cases (++) corresponded to a mean value of about one parasite per observation field.
- high positive infections (+++) were recorded when several or numerous parasites were present in each microscopic field.

2.2.2 Determination of infectious dose

Experimentation 1:

A first series of 3 similar experiments (A, B, C) was performed by injecting oysters (30 per group) with different numbers of purified parasites: 0; 1; 100; 10,000. The mortalities were monitored daily in order to look for the parasite in heart-smears. Cumulative mortalities, with and without *Bonamia*, were monthly expressed for each group (M1; M2; M3; M4). After four months, all the surviving oysters were sacrificed and examined for *B. ostreae*. Thus, T4 corresponded to dead plus sacrificed oysters, with or without *Bonamia*.

Experimentation 2:

This series of experiments was performed and monitored as previously described, with different numbers of purified parasites: 0; 100; 10,000; 1,000,000. Each experimental group consisted of about 30 oysters, excepted for the highest infection dose where 42 oysters were injected.

2.2.1.2 Kinetics of development of the bonamiasis

Each of 100 oysters was injected with 220,000 parasites obtained from a single purification. Mortalities were monitored daily and cumulative monthly totals calculatedcumulated (M1; M2; M3; M4). Some oysters were sacrificed every month during a four-month period (S1; S2; S3; S4).

2.2.2 Application in the assessment of relative resistant oysters

A series of experimental infections was performed as previously described with oysters from four different origins in France.

A first group (A) of 160 three year old oysters were reared from spat produced in a hatchery from old oysters collected in the chronically infected area of Quiberon Bay, and were thus considered as possibly "resistant". These experimental oysters were cultivated in Quiberon Bay.

Two other groups of oysters from the same area were used, one consisted of 43 three years old oysters (B) and the second of 97 four years old oysters (C).

The last experimental group (D) consisted of 61 three years old oysters collected from a natural oyster bed in the Penzé River, an area of very low infection. The oysters were injected with 100,000 purified parasites obtained from a single purification. Mortalities were monitored daily and cumulated bimonthly during a 6-month period (M0; M2; M4; M6). At two month intervals, a smear was prepared from gill biopsy (G2; G4; G6) for each surviving oyster to look for parasite.

2.2.3 Improvement of the protocol of experimental infection

Effect of stress on the development of bonamiasis

Two groups of oysters from Tralee, an uninfected area, were inoculated under the same conditions, with approximately 500 000 purified parasites in 100 µL Percoll plus filtered seawater per oyster. Group 1 were shell cut without anaesthesis to allow inoculation (n=70). Group 2 were anaesthetised without cutting to allow inoculation (n=70) and controls for each group received filter seawater instead of parasites (n=30 for each group).

Improvement of the protocol of experimental infection

Working session on the bonamiasis, 21 January- 1 March 91,(annexed program)

After a 3 hour bath in $MgCl_2$ (5%) for anaesthesia, Mediterranean oysters (30 per batch) were inoculated into the pericardial cavity with 0, 40000, 80000 and 120000 purified parasites in 50 µL filter seawater per oyster. The mortalities were recorded daily, to give cumulative monthly totals (M1, M2, M3, M4). At the end of the experiment, the oysters were sacrificed and analysed for the presence of *Bonamia*.

CHAPTER 3

RESULTS

3.1 FIELD TRIAL

The susceptibility of the four Irish oyster populations to *Bonamia* was tested.

3.1.1 Trial 1:

The prevalence of infection and cumulative mortalities of Cork, Galway and Belmullet oysters over 10 months are given in the figure 73. At the beginning of the trial in January 1990, Cork oysters showed 35% prevalence of infection and Galway and Bellmulet oysters were uninfected. In June, infection levels in the Cork sample peaked at 64.2% while infection levels in the other populations were quite low at 8.2% and 3.5%. However, by October, infection in the Cork group had dropped to 5% but with a cumulative mortality of 33.6%. Galway and Belmullet oysters had reached infection levels of 38.5% and 46.2%, with associated mortalities.
Thus, all three populations developed high prevalence of disease with significant mortality. The Cork oysters did not show evidence of resistance, however the course of the disease had already begun in Cork oysters at the start of the trial.

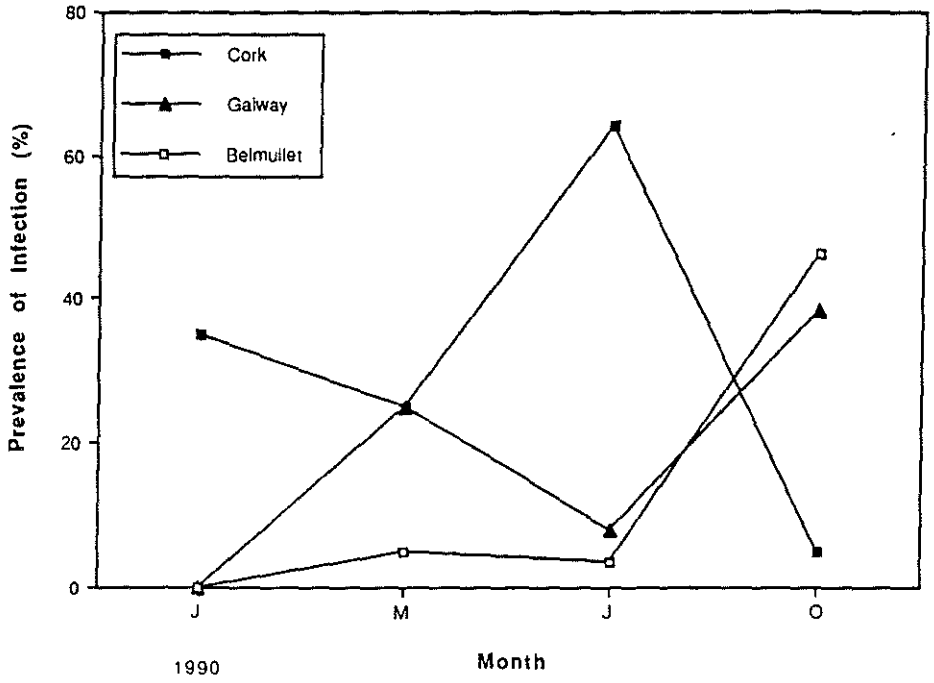
3.1.2 Trial 2:

Tralee oysters replaced the Belmullet ones. At the beginning of this trial, in April 1991, Cork oysters had a prevalence of infection of 30%, Galways oysters had 15% prevalence and Tralee oysters were uninfected (fig.74). By June the disease in Cork oysters had dropped to 0% with 7.1% mortality. Disease level in the Galway oysters had also dropped to 3.5% with high mortality. Tralee oysters had developed a low level of disease (1.7%). In October, a sample of Cork oysters was not available due to unforseen circumstances. Galway oysters had 32.1% prevalence of infection and Tralee oysters 60.3% and both had associated high cumulative mortalities (48.2% and 13.12% respectively).

3.1.3 Trial 3:

This trial, begun in November 1991, showed an initial prevalence of infection in the Cork and Galway groups of 9.6% and 26.3% respectively. Tralee oysters were uninfected at this date (fig. 75). After 3 months, in February, prevalence in the cork group hah risen to 24.6%, but in the Galway one had dropped to 10.7%. 7.7% of the Tralee oysters showed infection. In May, the prevalence of infection were in both the Cork and Tralee groups 12.1% and 5.2% respectively, and 15.5% in the Galway group whose % cumulative mortality rose the highest value of 45.8%.

A



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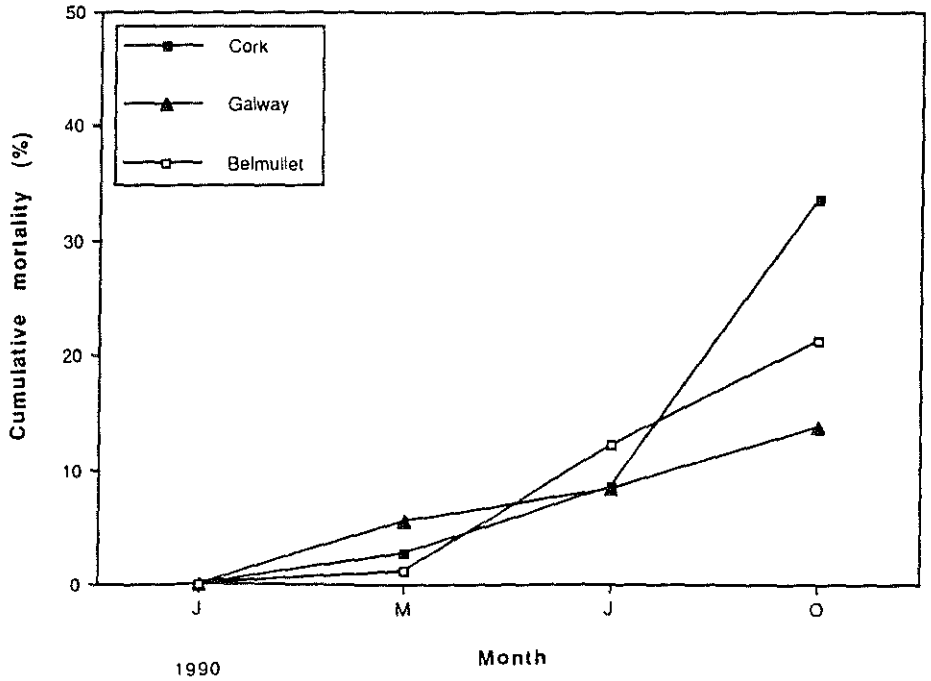

Figure 73. Prevalence of infection (A) and cumulative mortality (B) (%) in three Irish oyster populations in Field Trial 1.

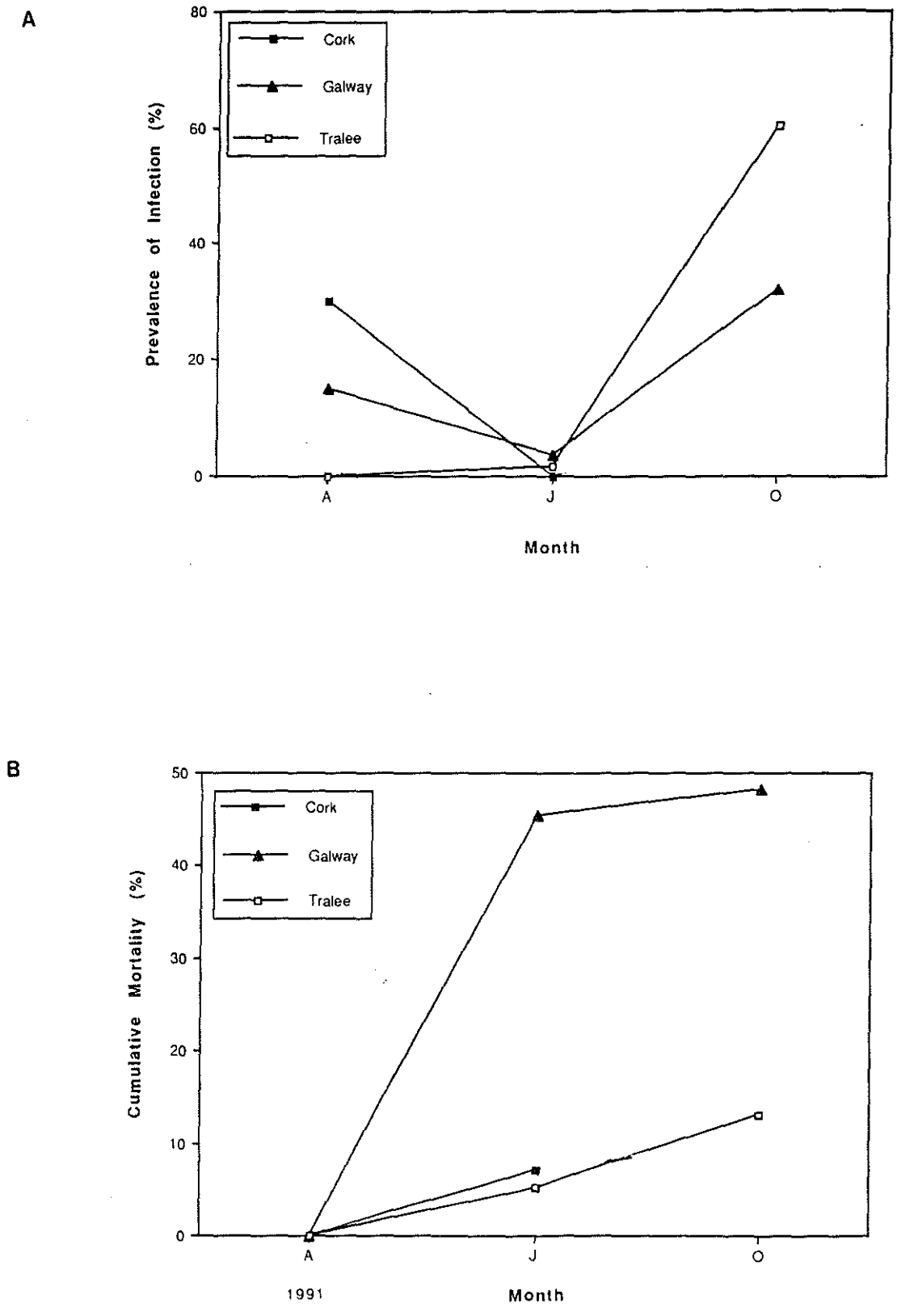

Figure 74. Prevalence of infection (A) and cumulative mortality (B) (%) in three Irish oyster populations in Field Trial 2.

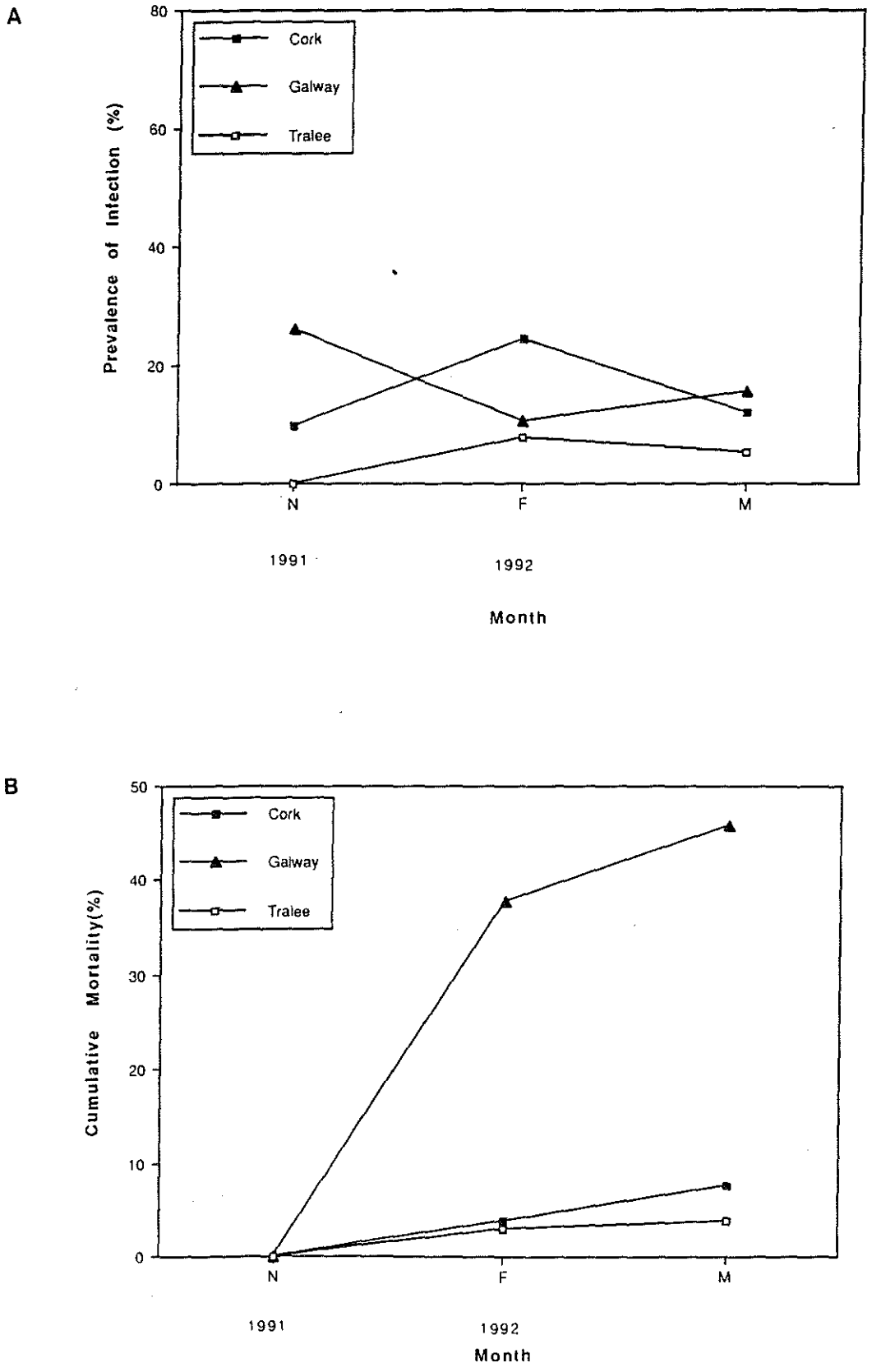

Figure 75. Prevalence of infection (A) and cumulative mortality (B) (%) in three Irish oyster populations in Field Trial 3.

3.2 LABORATORY TRIALS

3.2.1 Experimental infection protocol

3.2.1.1 Determination of infectious dose

Experimentation 1: The relation between infection dose levels and bonamiasis development was investigated in a first series of three similar experiments (A, B, C) by injecting oysters with respectively 0, 1, 100 or 10,000 purified parasites. Cumulative mortalities and infections are reported in figure 76.

The first observation concerned mortalities which occurred before the second month. These mortalities were not associated with *B.ostreae* infection and were considered as resulting from cutting the valves. After injection of one *B.ostreae*, only one case of infection was recorded in a single oyster sacrified after four months (experiment A). With an infectious dose of 100 parasites, a few oysters died between the second and third month, with low infections. Whereas these infections were patent, they could not be directly the cause of mortality. The highest tested doses, corresponding to 10,000 parasites, led to infections which were also detected after the second month. The infection rates were higher than with the lower doses, but yet relatively limited at around 28%. It appears that, from the third month, mortalities were related to bonamiasis development.

Experimentation 2: On the basis of these preliminary results, another experiment was performed to decrease early mortalities and to increase the infection rates. Care was taken to avoid mantle damage during valve removal. In addition to the previous infection dose levels (0; 100; 10,000 parasites per oyster), some oysters were also inoculated with 1,000,000 parasites. As shown in figure 77, early mortalities were reduced and were 11% and 20% respectively at the second and the fourth months in the controls. Infection levels recorded were similar to the previous experiments for the lowest doses. With the highest dose, one infection was detected as early as the second month. From the second month to the end of the experiment, most of the mortalities were associated with parasite infections. Moreover, during the fourth month the observed infection levels suggested that the mortalities were induced by the parasite. At the end of the fourth month, the surviving oysters were sacrified giving the final estimate of the infection rate of 69% in the group receiving the highest dose. On the basis of the infection rates obtained according to the dosage, the 50% infectious dose was estimated around 80,000 parasites per oyster by using the Reed and Meunch Method (131), illustrated in table 18.

3.2.2.2 Kinetics of bonamiasis development

In the aim of analysing the kinetics of bonamiasis, this experiment was undertaken with 100 oysters in order to have sufficient oysters to monitor mortalities and also for monthly sampling of live oysters. Each oyster was inoculated with 220,000 purified parasites in order to obtain a high infection rate. Figure 78 shows the respective monthly results for mortalities (M) and sacrificed oysters (S).

After the first month, four oysters had died and none showed infection with *B.ostreae*, whereas one positive case was found among 21 oysters which were sacrificed. As in the previous experiments, infections became evident from the second month. The apparent infection rates estimated monthly from mortalities were respectively 0% (M1), 38% (M2), 79% (M3) and 100% (M4) whereas the estimations from sacrificed oysters at the same times were 5% (S1), 50% (S2), 100% (S3) and 100% (S4).

Thirty five of the 40 surviving oysters (M3, S3; M4, S4) at the end

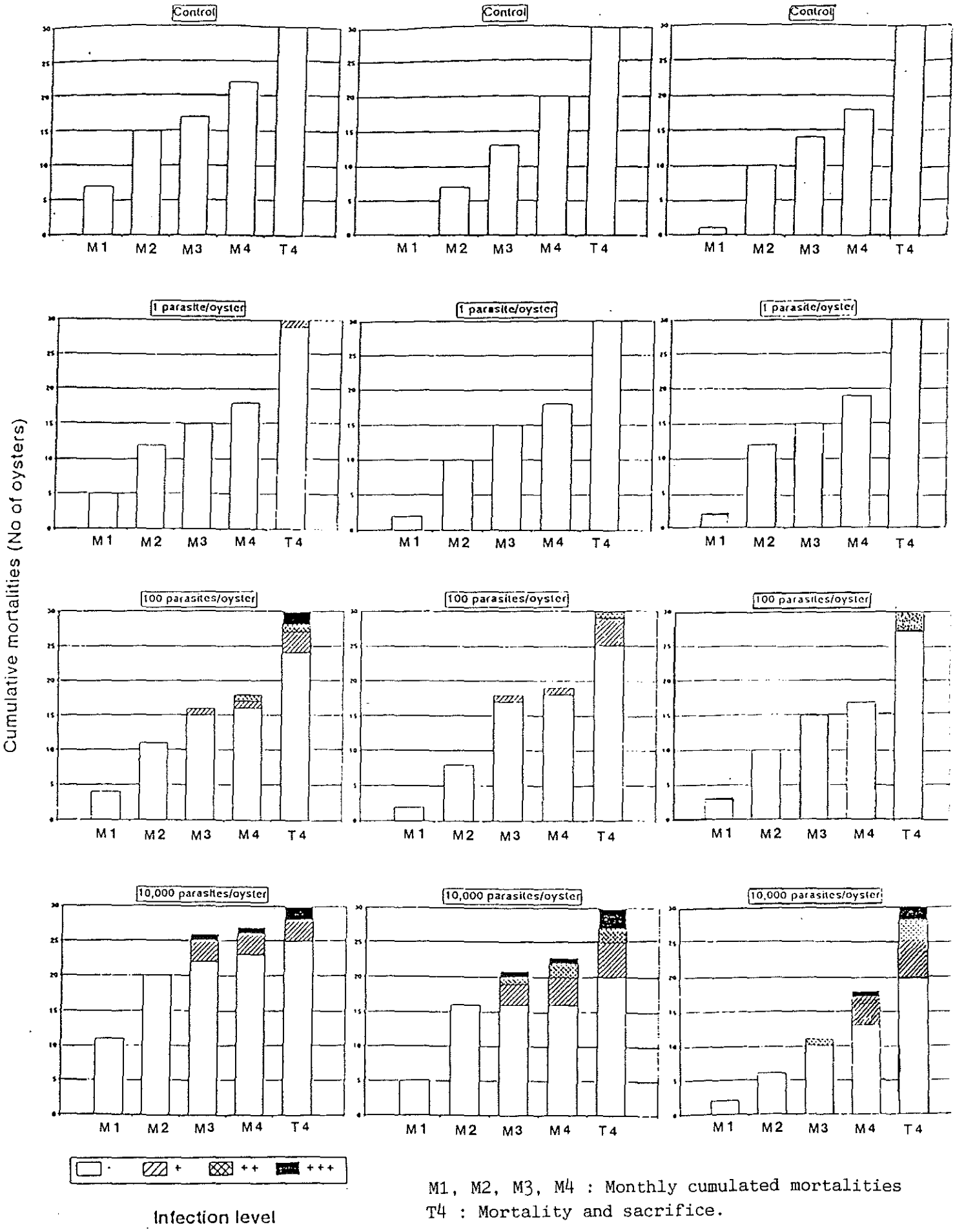

Figure 76. Relation between infection dose levels and bonamiasis development (Experimentation 1).

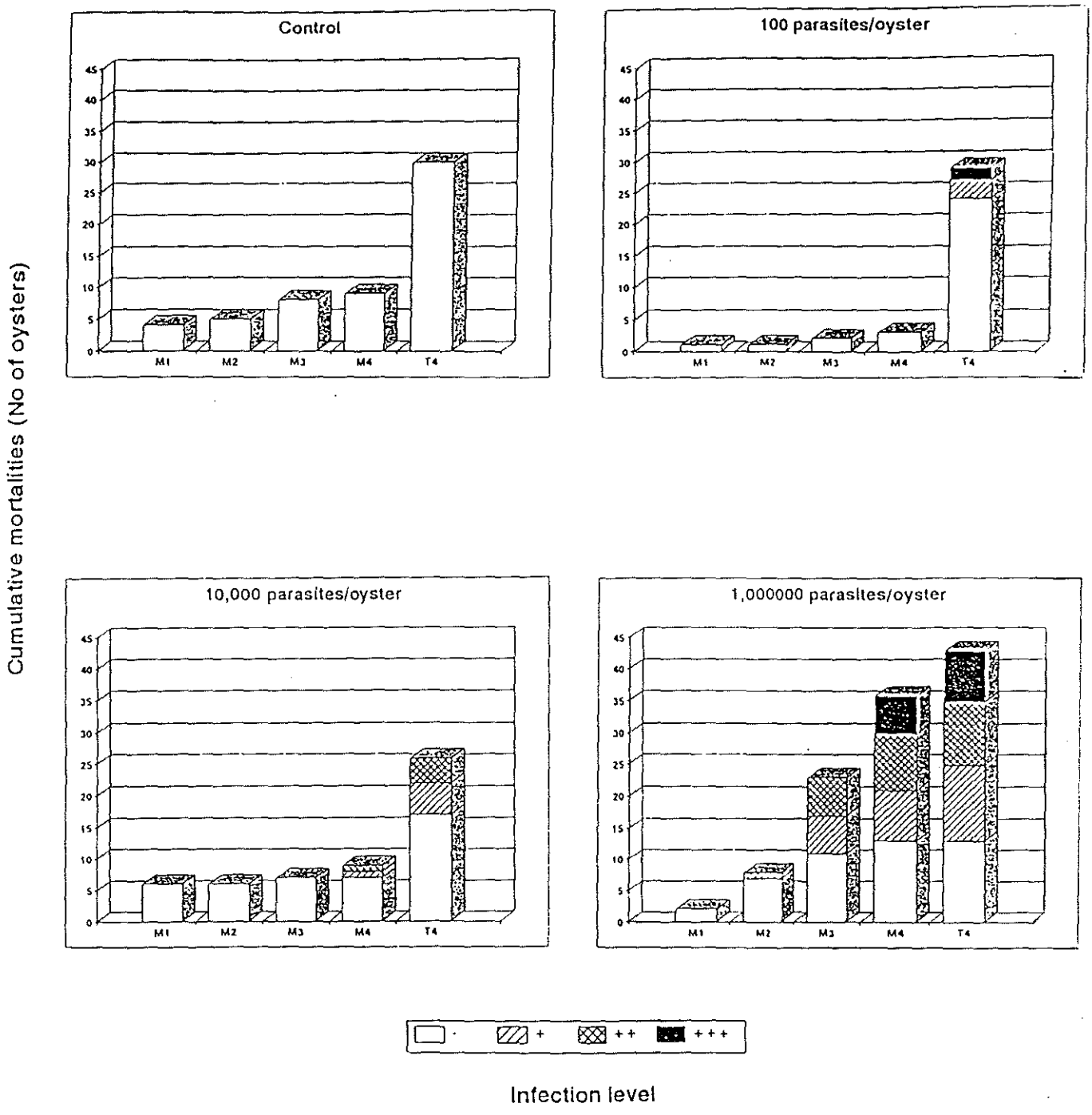

Figure 77. Relation between infection dose levels and bonamiasis dévelopment (Experimentation 2).

M1, M2, M3, M4 : Monthly cumulative mortalities
T4 : Mortality and sacrifice.

No of parasites per oyster	A		B		% of infected oysters
	No of oysters				
	I	NI	I	NI	
10^6	30	13	44	13	77
10^5	0	0	14	13	52
10^4	9	17	14	30	32
10^3	0	0	5	30	14
10^2	5	24	5	54	8
10^1	0	0	0	54	0

I: infected oysters
NI: non infected oysters

The numbers in column B were obtained by successive addition of the numbers in column A, from the bottom to the top for infected oysters and from the top to the bottom for non infected oysters.
The 50% infectious dose (50% ID) was calculated from the proportional distance between the two numbers close to 50%.

$52 - 50 / 52 - 32 = 0.1$

$10^{5-0,1} = 10^{4,9}$ parasites / oyster

50% Infectious Dose = 79,430 parasites / oyster.

Table 18. - 50% infectious dose determination according to the Reed and Meunch Method.

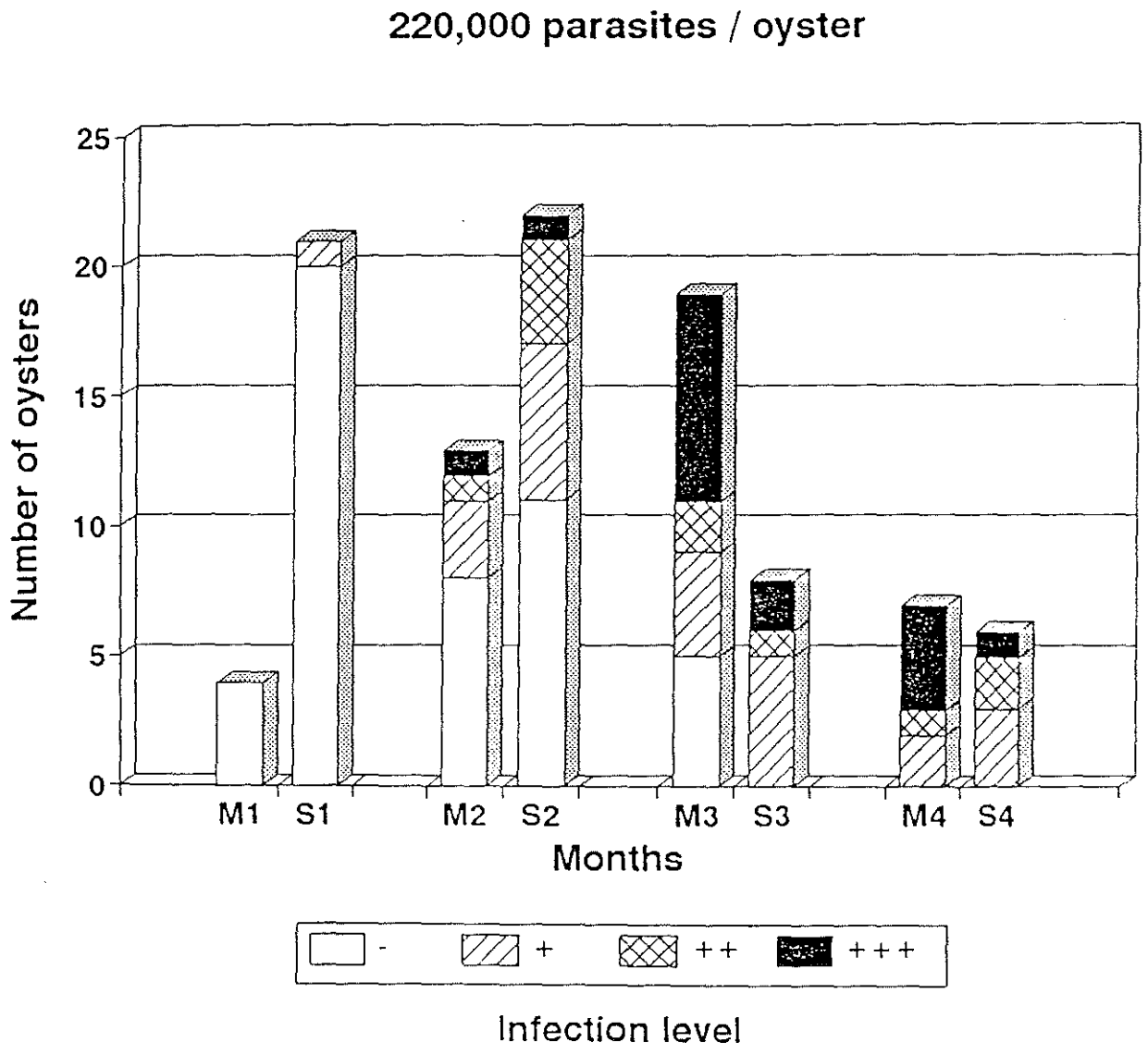

Figure 78. Kinetics of bonamiasis development.

of the second month were infected at a rate of 87%. Thus some oysters were falsely identified as negative at the second sacrifice (S2) since infection rate was estimated at only 50%. These discrepencies suggested an insufficient sensitivity of the histological diagnostic method.

These results demonstrated individual variability of bonamiasis kinetics since, for the same infectious dose, some oysters showed a high infecton as early as the second month whereas cases of low infection were still observed at the fourth month.

3.2.2 Assessment of the sensibility of different oyster strains to *B. ostreae*

Because experimental infections could be standardized, the respective sensitivity of flat oysters to *B.ostreae* from different origins was compared. Four groups were identified: A- Quiberon F1 oysters possibly "resistant"; B- Quiberon three years old oysters; C- Quiberon four years old oysters; D- Penzé river 3 years old oysters.

In each of the groups, non-infected oysters were initially selected on the basis of a negative examination of a smear prepared from gill biopsy (G0). Each animal was inoculated with 100,000 parasites, that was previously demonstrated to be about the 50% infectious dose for three years old flat oysters from Mediterranean Sea. Results are reported in the figure 79.

Very few early mortalities were observed in any of the experiments. From the second month, the majority of mortalities were associated with parasite infection. After six months, cumulative mortalities (M6) were equivalent to 32.5% in group A whereas in the other groups (B, C, D) they were respectively equivalent to 76.7%, 74% and 78.6%. The final survival rate (G6) was the highest in oyster group A, where the infection rate was the lowest.

It must be emphasized that for research into the parasite, the gill-biopsy technique constituted an interesting experimental improvement because it avoided sacrificing the animals for parasite analysis.

Moreover it must be noted that the diagnostic method may be insufficiently sensitive since in all experiments, only a few positive cases were detected at the second month and some animals still appeared falsely-negative at the fourth month.

3.2.3 Improvement of the infection protocol

- <u>Shell-cutting as a stressor influencing the development of bonamiasis</u>

Laboratory-based tests that involved inoculation of parasites, had initially relied on cutting of the oyster's shell to facilitate inoculation. However this method could be considered traumatic and a stressor for the oysters, so exacerbing the sensibility to the parasites. Following the development of suitable anaesthetic for the flat oyster, an experimentation was carried out to compare the effect of the two methods (shell-cutting and anaesthesis) on the development of the disease.

Table 19 shows the prevalence of infection and percentage mortality in each group at the end of the experimental period (12 weeks). Prevalence of infection in the shell-cutting (cut)/inoculated group was 8.0% and in the anaesthetised/inoculated group 4.0%, with 68.6% and 41.4% mortality respectively. Thus prevalence of infection and mortality were marginally higher in the group whose shells were cut.

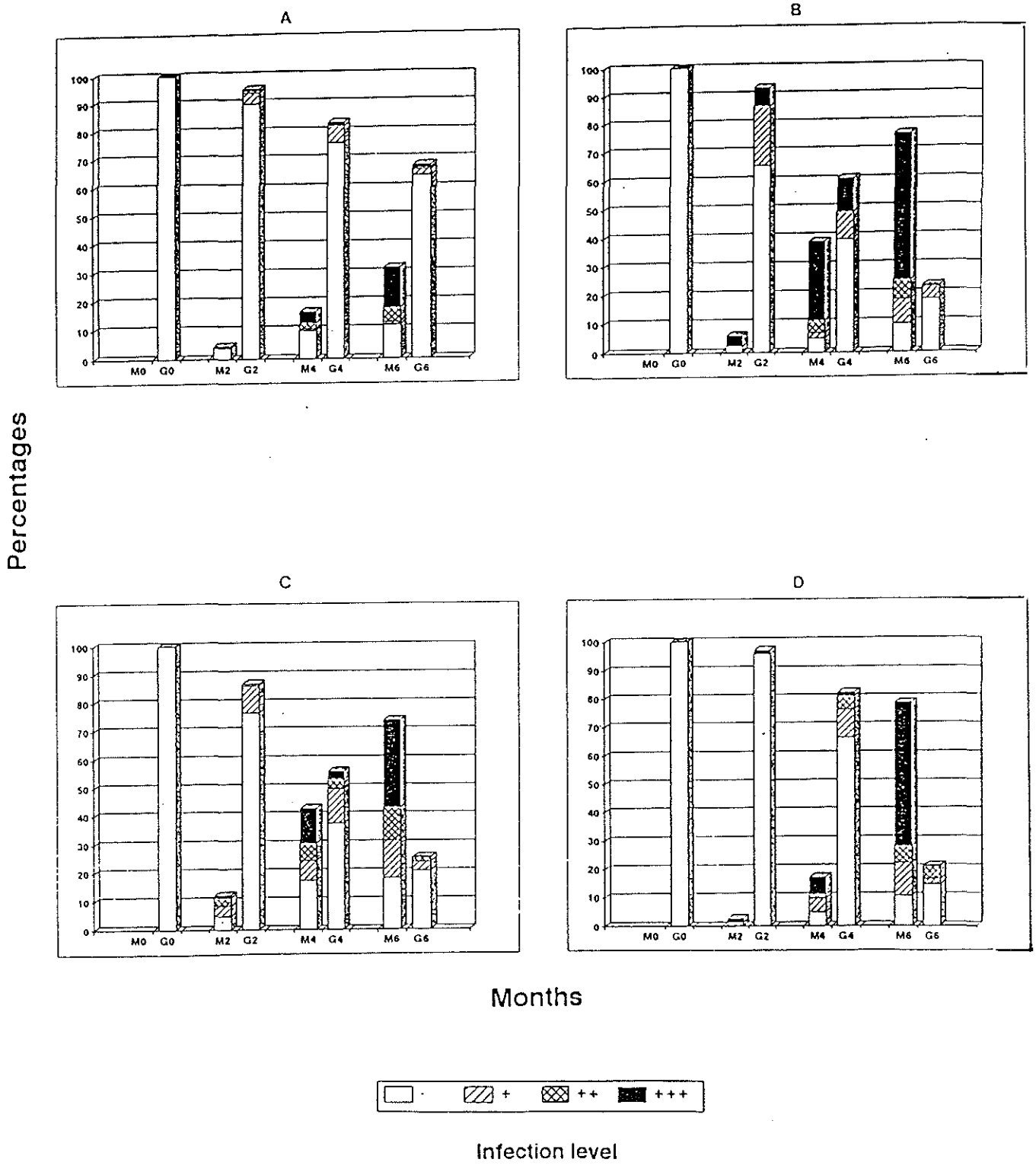

Figure 79. Comparison of sensitivity to *B. ostreae* between different flat oysters strains. A : Quiberon F1, B : Quiberon 3 yars old, C : Quiberon 4 years old, D; Penze River 3 years old oysters.

	Anaesthetised inoculated	Cut shell inoculated	Anaesthetised FSW	Cut shell FSW
%Prev.	4.0	8.0	0.0	0.0
%Mort.	41.4	68.6	3.3	90.0*

* This mortality % can be due to a water quality deteriorated.

Table 19. Prevalence of infection (%) and (%) mortality in two control groups twelve weeks post-inoculation.

These results would indicate that oysters whose shells were cut and which were inoculated with Bonamia had higher mortalities and prevalence of infection than other groups.

-Improvement of the infection protocol

It appeared necessary to improve the protocol of experimental infection, particularly by using less traumatic methods for the injection of the parasites.

Firstly, the valvotomy was replaced by an anesthesia of the oysters. The magnesium chloride, at the concentration of 5% (W/V) in sea water, constitute a good oyster anesthetic (132). Moreover, the opening of the oyster valves permitted to inoculate the parasites in the pericardial cavity, without damage, instead of the digestive gland.

Two months post-inoculation, the early mortalities due to the inoculation were greatly reduced to 6% and 4 months post-inoculation, mortalities were associated with *Bonamia* for the three infectious doses (fig. 80).

At the end of the experimentation, the cumulated percentages of infection were respectively 0%, 40.7%, 44% and 53.3% for the infectious doses of 0, 40 000, 80 000 and 120 000 parasites per oyster. According to the method of Reed and Meunch (131), the infectious dose 50% was estimeated to 94 560 parasites per oyster.

3.2.4 Control of the refractoriness of *Crassostrea gigas* to *B. ostreae*

In order to verify at laboratory the refractoriness of the Japaneese oyster to *Bonamia*, which is ascertained in the field, 30 C. *gigas* were inoculated with 500 000 purified parasites as previously described. Four months post-inoculation, no mortality was recorded for *C. gigas*, when inoculated O. edulis displayed cumulated infection rate of 56.6%.

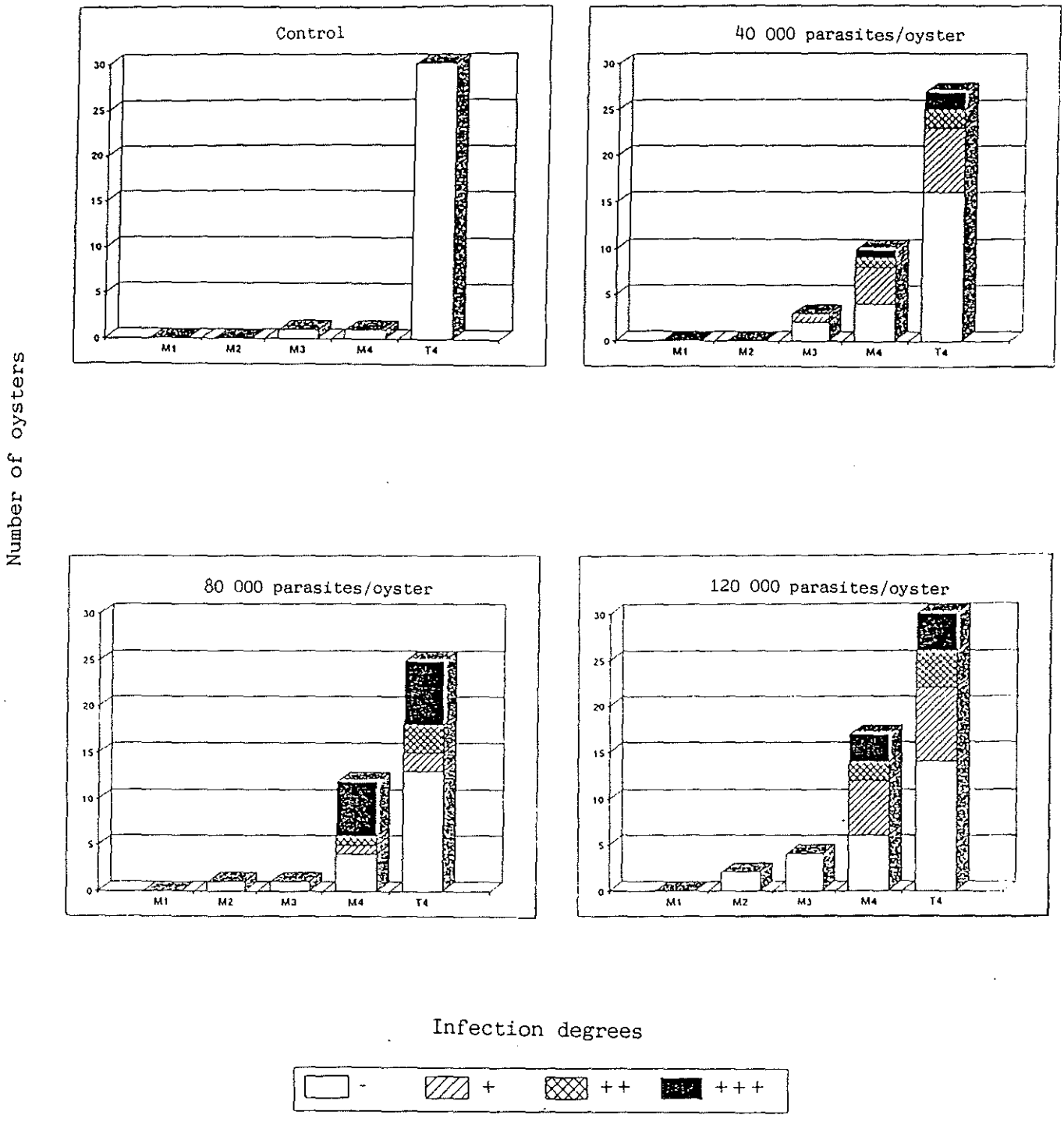

Figure 80. Relationship between the quantity of inoculated parasites and the development of the bonamiasis - inoculation in pericardial cavity.

M1, M2, M3, M4 : Monthly cumulative mortalities
T4 : Mortality and sacrifice.

CHAPTER 4

DISCUSSION

The selection of resistant oysters can be viewed as a long term approach to reduce the impact of bonamiasis from an economical standpoint. Two approaches can be adopted. One is based on the identification of surviving animals in the field. The second way consists of exerting, on surviving animals and their offspring, a strong and constant pressure. However this approach depends on availability and reliability of techniques of experimental pathology.

This study, firstly, set out to look at the relative susceptibility of several oyster populations to *Bonamia*. They came from Cork, Galway, Tralee and Belmullet in Ireland. All oysters employed for each trial were the same age. However, Cork oysters were coming from a diseased area and so had been exposed to the parasite throughout their life cycle. They showed a certain prevalence at the beginning of each trial. Galway oysters were deemed disease-free and those from Tralee and Belmullet were uninfected at the beginning of the assays. The areas they came from continue to be considered disease-free. During the trials, the various oyster groups showed varying patterns of disease. The trend was for increasing prevalence initially with gradually increasing mortality, leading subsequently to lower prevalences among the survivvors. There is no consistent evidence from any of the three field studies that Cork oysters are more resistant to the disease than oysters from other locations.

Resistance is considered acquired through exposure to the parasite."The development of resistance in molluscan shellfish populations as a consequence of massive disease-caused mortalities is indicated by a gradual decline in mortalities despite a continual high prevalence of infections" (133). Experience of development of resistance e.g. in MSX and Malpeque disease would indicate that long time periods would be required (134;135). The experience of resistance selection to MSX in *C. virginica* has been taking place over many years through selective breeding in the laboratory and the field. In MSX, the ability to tolerate chronic infections increased with selective breeding but was limited, and eventually all oysters were debilitated beyond the point of recovery but many reached marked size before this (134;138). Moreover, high death rates were observed in the F4 and F5 generations, probably caused by inbreeding, bottlenecks and/or intense selection for a single trait (136).

Hybridization of two types of oysters could be one method of combining desirable genes (137). Haskin and Ford (138) in an attempt to decrease susceptibility to MSX, crossed wild stocks of *C. virginica* from different locations, inbred selected strains and crossed inbred strains and members of the parent wild stock. But there was no evidence to suggest that the first two outcrosses were more resistant.

The difficulties and failures in the selection of MSX resistant oysters based on trial assays can be essentially explained by the impossiblity of exerting selection pressure. Indeed these studies were based on natural infections which, occurring in the field, fluctuate and do not stabilize resistant characteristics (141). Therefore in the present study of bonamiasis, one of the top priorities was to develop and standardize the experimental reproduction of the disease.

Following the preliminary results obtained for bonamiasis with infected oyster homogenates introduced into aquaria and with infected haemocytes injected into oysters (139) the establishment of an experimental

protocol with reproducible results was undertaken. Such a protocol was dependent on the availability of purified parasites (11).

Initially, it was necessary to develop the inoculation method. The accessibility of tissues was firstly based on partial valve removal, which was easy to perform, but which required caution to avoid mantle disruption and subsequent septicaemia and mortalities. Several injection sites were tested. The digestive gland was tested because of its proximity to the valve gap and the importance of its connective tissue containing haemocytes. The branchial efferent vein was also accessible with a syringe, but heavy bleeding resulted from the injection.
In practice, injections were easily made into the digestive gland, although artefacts may have resulted from parasite inoculation into the caecum. Moreover, septicaemia could result from disruption of the digestive epithelium, so control oysters were similarly inoculated with sterile sea water.

The effect of the infectious dose was initially investigated with a range of parasites from 1 to 10 000. It appeared that one parasite can rarely induce an infection and it was surprisingly observed that, even with 10 000 parasites per oyster, the prevalence was relatively low (less than 40%). Moreover, the histological analyses, particularly those performed on sacrified oysters, showed large differences in the infection levels, suggesting an individual variability in oyster response to *B.ostreae*.

After that, very high infectious doses were tested (1 million parasites per oyster), confirming the previous data but moreover permitting the determination of the 50% infectious dose (50% ID) for a four-month-experiment. Thus, 80 000 parasites were necessary to induce bonamiasis in three years old oysters from the Mediterranean Sea. The possible evaluation of 50% ID for oyster samples permits a relatively objective comparison between the *B.ostreae* sensitivities of different oyster strains. It must be reminded that previous data were published from experimental infections performed by proximity (140), but *Bonamia*-sensitivities of oyster strains were only compared and not intrinsically estimated.

From an experimental point of view, infectious doses ranging from 100,000 to 1 million per oyster would lead to significant prevalences and variable infection levels. This individual variability of infection levels resulting from the same infectious dose was confirmed in the experiment designed to investigate the kinetics of *B.ostreae* development. Two major observations merit discussion. Firstly, during the initial period following experimental infection, detection of the parasite by histology is practically impossible because of the lack of sensitivity of the method. This fact must be kept in mind during epidemiological surveys for the disease, since oysters in the early stages of infection could be recorded as uninfected. Thus, more sensitive diagnostic methods such as PCR (Polymerase chain Reaction), would be usefull. Moreover, the variable development of *B.ostreae* in individual oysters would suggest the involvement of an immune response.

From these experiments, it apppeared necessary to improve the protocol of reproduction of the bonamiasis, particularly the method for getting to the oyster tissues and inoculating the parasites. Indeed, high % post-inoculation mortality suggested a significant impact by shell-cutting resulting in possible stress or scepticemia. So, a comparative assay was carried out by inoculating *Bonamia* in oysters either shell-cutted or anaesthetised. Shell-cutting appeared to have effectively led to an increased susceptibility to *Bonamia* and in increased mortality. Moreover, the use of anaesthesia made it possible to inject the parasites into tissues other than the digestive gland. The well differentiated pericardiac cavity, containing numerous circulating haemocytes was chosen. The assays

effectively showed low early mortalities.

Finally, on the basis of experimental infection assay, the refractoriness of C. gigas was also demonstrated at animal level.

In conclusion, the exploitation of this experimental protocol for reproducing bonamiasis in flat oysters could open the way for genetic research with resistant oyster selection. The preliminary results obtained showed that the strain which is relatively less sensitive to *B.ostreae* was the F1 generation from possibly resistant oysters collected in a chronically infected area. Thus, for breeding programs, quantitative geneticists could work in laboratories by applying permanent and strong selective pressure and by identifying the most resistant oysters.

GENERAL CONCLUSION

Since the appearence in 1979, of *Bonamia ostreae*, in South Brittany (France), the disease has extended to the main breeding areas of France, excepted Mediterranean, and to several European countries such as Britain, Ireland, Holland and Spain. Moreover, cases of bonamiasis have been identifed on the west coast of USA (15) and also in New Zealand (Dinamani et al., 1987).

Recent epidemiological studies have shown that this endemic disease affects all the areas of intensive breeding. Prevalences of *Bonamia ostreae* are extremely variable, depending upon the area, the oyster age and period of the year. In Galicia (Spain), the prevalences reach 73% with an average value of 43%. In France, the epidemiological status of bonamiasis remains stable with average prevalences of about 6% but with cases of higher infection rates of 20% to 32%, linked to handling and transfers of the oysters. In Ireland, one of the most important areas (Cork Harbour) is heavily infected (45% of infection), and the disease recently appeared in some wild stocks areas.

In order to reduce the impact of the bonamiasis, zootechnic measures can be established, based on knowledge of the parameters promoting development of the disease. The aim of analytical epidemiology studies is to take into consideration the respective influence both of environmental parameters and the stressor factors related to the zootechnical practices.

With regard to zootechnology, the impact of oyster transfers and handling but also high densities of animals, have been implicated in the development of bonamiasis. Whichever the culture method, on bottom or raft suspended, the parasite spreads. However, in the Mediterranean, flat oysters are farmed on rafts and, though oysters from infected areas are thought have been been introduced to the Mediterranean, until now very few cases of *Bonamia* infection have been detected, and the disease has not spread. Some undetermined parameters such as high salinities, could be involved more than culture methods. Zootechnic practices can be adapted in order to limit the stresses leading to bonamiasis. Densities of oysters can be reduced and transfers can be planned with regard to the breeding period leading to a weakening of the oysters, and taking into account the presence of *Bonamia*. Unfortunately, environmental factors cannot be controlled. However, the ability to recognise environmental stress as a pre-disposing factor in the occurence of the disease, is necessary to manage oyster culture and for zoosanitary controls.

The work of Hawkins and Hutchinson is of fundamental importance. "This investigation has incorporated and extended the stress assessment methods and rationale set out by Bayne *et al.*(*The effects of stress and pollution on Marine Animals. Praeger, New-York. 384pp.,1985*) to establish the effects of various forms of environmental stress on *O. edulis*. The three environmental parameters to be investigated were water température, salinity and tidal exposure. Particular emphasis has been laid on the detailed examination of these effects which may reveal stress induced changes in the animals'physiology, biochemistry and immune systems that render them susceptible to pathogens."

It has been shown that some metabolic changes related to environmental factors could affect the haemocyte activity and consequently the disease susceptibility. So, several parameters related to defence reactions of the oysters have been considered to determine characteristics

which could be used as direct measures of immune capacity. In these studies, questions have been raised concerning the great variability of haemocyte count observed in the oyster populations. As shown during the working session on bonamiasis, the Quiberon F1 strain displayed significantly higher cell counts than other flat oyster strains. However, continuing research at the population level, will provide understanding about the significance of this parameter and about a possible genetic origin of the cell count.

In fact, progress in understanding of the disease development and the establishment of prophylactic measures, greatly depends on the concomitant establishment of indices for biochemical, physiological and immunological states of oyster populations. Beyond a more fundamental interest for the knowledge of oyster defence mechanism, the technique of chemiluminescence has been adapted to quantify the phagocytosis activity of the oyster haemocytes. Indeed, in vertebrates, this method is currently used to estimate the physiological and immunological state of animals. It was tested, in *Crassostrea gigas*, to assess the effect of some pollutants on the phagocytosis of the haemocytes. However, experiments at the population level are now required to validate the chemiluminescence as a immunological index.

Prophylactic measures which can be established depend on data related to the development of the diseases, acquired in the field. Mostly, they depend on knowledge of the disease and on the study, at the animal level, of the parasite biology, its transmission and development mode. Accordingly, work has been undertaken to purify of the parasite, because of the lack of molluscan cell lines and the impossibility to reproduce *Bonamia in vitro*. The availability of purified parasites has permitted on one hand the preparation of specific molecular probes, such as polyclonal or monoclonal antibodies which could be useful tools for diagnosis, and on the other hand, the establishment of experimental infections. This last application of *Bonamia* purification which is new in marine bivalves, has opened the way for studying the development kinetics of the protozoan, and locating the primary infection site.

So, following histological examination of the tissues of experimentally infected oysters, first signs of disease were seen about 35 days post-inoculation, confirming a latent period during which no parasite can be easily detected. This confirms the limits for diagnosing early stages of infection strictly by histological examination of tissues. It is also instrumental in demonstrating unequivocally that it is always hazardous to transfer animals in healthy areas, animals which can be wrongly diagnosed as *Bonamia*-free. Other, more sophisticated methods might be developed such as the Polymerase Chain Reaction technique, based on nucleotide hybridization. The high sensitivity of this method could be especially useful for detecting early disease stages in molluscan tissue samples.

In other respect, the refractoriness of *Crassostrea gigas*, the sympatric oyster species, has been definitively and experimentally verified. Several months after the inoculation of *Bonamia*, no mortality occured and no parasites could be detected in the sacrificed *C. gigas*. This result tends to remove suspicions about its role as a latent carrier

Besides prophylaxis based on diagnosis, another way to ensure uninterupted production relies upon the selection of resistant animals which could obviously be more effective. Such a strategy needs to identify animals displaying lower sensitivity to the parasites and then to effect constant and strong selection pressures. With the establishment of

experimental infections, these conditions are now satisfied. Indeed, a selection program is now being undertaken by IFREMER (France) based on the hatchery production of a strain of survivors from Quiberon Bay (South Brittany). The first generation (F1) was experimentally infected and displayed better survival than other inoculated control oysters (about 72% to 94%, and 46% to 66%, respectively). Now, the survivors of the selected F1 oysters will be used to produce a second generation (F2).

Selection of animals surviving natural infection in the field can be hazardous. In New-Jersey (Cheasepeake Bay), the selection program of *Crassostrea virginica* resistant to MSX (*Haplosporidium nelsoni*) has failed (141). Indeed, natural infections, largely depending on environmental factors, are very variable and so do not allow the stabilization of resistant features.

Experimental reproduction of the disease has revealed great individual variability of infection rates, suggesting different degrees of defence reactions. Taking into account the obligate intracellular nature of the protozoan, host-parasite interactions have been studied at the cellular level, looking for the functions of the haemocytes. The availability of purified parasites and haemocyte primary culture methodology led to elaborate a protocol of *in vitro* infection of the haemocytes. So, the processes of recognition and entry of *Bonamia* into the host cell were identified, showing a same similarity of phagocytosis events between the two oyster species, the definitive host, *O. edulis*, and the refractory *C. gigas*. Consequently, this led to the study of post-phagocytic events, with the aim of analysing, simultaneously the adaptation of *B. ostreae* to *O. edulis* haemocytes and the resistance of the *C. gigas* haemocytes. The microbicidal mechanisms already known in vertebrate phagocytes, the oxygen-dependent cytotoxic mechanism was investigated in bivalves and studied by the technique of chemiluminescence. After several *in vitro* chemiluminescence assays, it appeared that *B. ostreae* was able to avoid or counteract the oxidative microbicidal system of the haemocytes in both of these oyster species.

In addition, the role of some haemolymph components, involved in defence reactions, such as the antibacterial lysozyme and agglutinins, was studied by comparing healthy and naturally infected oysters. However, no correlation was found with *Bonamia* infection.

The question of the specificity barrier and of the elimination mechanism of *B. ostreae* in *C. gigas* remains unresolved. However, other microbicidal activities might be investigated at cellular and at molecular levels, developing *in vitro* assays better adapted to identify mechanisms against infection.

REFERENCES

REFERENCES

1 - PICHOT Y., COMPS M., TIGE G., GRIZEL H., RABOUIN M.A., 1980. Recherches sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L.. Rev. Trav. Inst. Pêches marit., 43: 131-140.

2 - COMPS M., 1983a. Recherches histologiques et cytologiques sur les infections intracellulaires de mollusques bivalves marins. Thèse de Doctorat, d'Etat en Sciences, Université de Montpellier, pp 128.

3 - BUCKE, D. 1988. Pathology of Bonamiasis. Parasitology Today, 4 (6): 174-176

4 - COCHENNEC N., HERVIO D., PANATIER B., BOULO V., MIALHE E., ROGIER H., GRIZEL H. and PAOLUCCI F., 1992. A direct monoclonal antibody sandwich immunoassay for detection of Bonamia ostreae (Ascetospora) in haemolymph samples of the flat oyster Ostrea edulis (Mollusca: Bivalvia). Dis. aqu. Org., 12: 129-134.

5 - ROGIER H., HERVIO D., BOULO V., CLAVIES C., HARVAUD E., BACHERE E., MIALHE E., GRIZEL H., PAU B., PAOLUCCI F., 1991. Monoclonal antibodies against *Bonamia ostreae* (Protozoa: Ascetospora), an intrahaemocytic parasite of flat oyster *Ostrea edulis* (Mollusca: Bivalvia). Dis. aqu. Org., 11: 135-142.

6 - BUCKE, D. AND FEIST, S., 1985. Bonamiasis in the flat oyster Ostrea edulis with comments on histological techniques. In Fish and Shellfish Pathology. Acad. Press.

7 - VAN BANNING, P., 1990. The life cycle of the oyster pathogen Bonamia ostreae with a presumptive phase in the ovarian tissue of the European flat oyster, Ostrea edulis. Aquaculture, 84: 189-192.

8 - TIGE G. and GRIZEL, H., 1982. Essai de contamination d'*Ostrea edulis* Linné par *Bonamia ostreae* (PICHOT *et al.*, 1979) en rivière de Crach (Morbihan). Rev. Trav. Inst. Pêches marit., 46 (4), 307-314.

9 - HINE, P.M., 1991. The annual pattern of infection by *Bonamia* sp. in New Zealand flat oysters *Tiostrea chilensis*. Aquaculture, 93, 241-251.

10 - MC ARDLE J.F., MC KIERNAN F., FOLEY H, JONES DH, 1991. The current status of *Bonamia* disease in Ireland. Aquaculture, 93: 273-278.

11 - MIALHE E., BACHERE E., CHAGOT D., GRIZEL H., 1988. Isolation and purification of the protozoan *Bonamia ostreae* (Pichot *et al.* 1980), a parasite affecting the flat oyster *Ostrea edulis* L.. Aquaculture, 71: 293-299.

12 - HERVIO D., 1988. Adaptation de *Bonamia ostreae* (Ascetospora) à la survie intra-hémocytaire chez l'huître plate *Ostrea edulis* L.. Etudes *in vitro* et *in vivo*. Rapport de DEA, Option Protistologie, Université de Clermont-Ferrand II, pp. 32.

13 - FIGUERAS A.J., 1991. *Bonamia* status and its effects in cultured flat oysters in the ria of Vigo (N.W. Spain). Aquaculture, 93, 225-233.

14 - GRIZEL, H. and TIGE, G., 1982. Evolution of the haemocytic disease

caused by *Bonamia ostreae*. 3rd Int. Coll. Invert. Pathol. 6-10 sept., Brighton.

15 - ELSTON R.A., FARLEY C.A., KENT M.L., 1986. Occurrence and significance of bonamiasis in european flat oysters *Ostrea edulis* in North America. Dis. aqu. Org., 2: 49-54.

16 - MALEK, E.A. and CHENG, T. (Eds.), 1974. In Medical and Economic Malacology. Academic Press, New York.

17 - BAYNE, B. L., BROWN, D. A., BURNS, K., DIXON, D. R., IVANOVICI, A., LIVINGSTONE, D. R., LOWE, D. M., MOORE, M. N., STEBBING, A. R. D. & WIDDOWS, J. 1985. The Effects of Stress and Pollution on Marine Animals. Praeger, New York. 384pp.

18 - WALNE, P.R., 1972. The influence of current speed, body size and water temperature on the filtration rate of five species of bivalve. Journal of the Marine Biological Association of the U.K., 52, 345-374.

19 - WALNE, P.R., 1979. Culture of Bivalve Molluscs. 2nd Edition, Fishing News Books Ltd, Farnham.

20 - WINBERG, G. E., 1960. Rate of metabolism and food requirements of fishes. Fish. Res. Bd. Can. transl. Ser. 194, 1-253.

21 - WARREN, G. E. & DAVIS, G. E., 1967. Laboratory studies on the feeding, bioenergetics and growth of fish. In: The biological basis of freshwater fish production, ed. S. D. Gerking, Blackwell Scientific Publications, Oxford, pp.175-214.

22 - SOLORZANO, L., 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr., 14, 799-801.

23 - DOERING, P.H. & OVIATT, C.A., 1986. Application of filtration rate models to field populations of bivalves: an assessment using experimental mesocosms. Marine Ecology Progress Series, 31, 265-275.

24 - BAYNE, B.L., HAWKINS, A.J.S. & NAVARRO, E., 1987. Feeding and digestion by the mussel *Mytilus edulis* L. (Bivalvia:Mollusca) in mixtures of silt and algal cells at low concentrations. Journal of Experimental Marine Biology and Ecology, 111, 1-22.

25 - JORGENSEN, C.B., 1990. Bivalve Filter Feeding: Hydrodynamics, Bioenergetics, Physiology and Ecology. Olsen & Olsen, Denmark. 140 pp.

26 - MOHLENBERG, F. & RIISGÅRD, H.U., 1978. Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia, 17, 239-246.

27 - YONGE, C.M., 1926. Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. Journal of the Marine Biological Association of the U.K., 14, 295-386.

28 - FOSTER-SMITH, R.L., 1975. The effect of concentration of suspension on filtration rates and pseudofaecal production for *Mytilus edulis* (L)., *Cerastoderma edule* (L) and *Venerupis pullastra* (Montagu). J. exp. mar. Biol. Ecol. 17, 1-22.

29 - HILDRETH, D. I. & CRISP, D. J., 1976. A corrected formula for calculation of filtration rate of bivalve molluscs in an experimental flowing system. J. Mar. Biol. Assoc. U. K., 56, 110-120.

30 - CONOVER, R. J., 1966. Assimilation of organic material by zooplankton. Limnol. Oceanogr. 11, 338-354.

31 - WIDDOWS, J., FIETH, P. AND WORRALL, C.M., 1979. Relationships between seston, available food and feeding activity in the common mussel *Mytilus edulis*. Mar. Biol., 50, 195-207.

32 - IVANOVICI, A. & WIEBE, W. J., 1981. Towards a working definition of 'stress': A review and critique. In: Stress Effects on Natural Ecosystems, eds. G. W. Barrett and R. Rosenberg, Wiley-Interscience, New York. pp. 13-27.

33 - ATKINSON, D. E., 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry, 7; 4030-4034.

34 - GABBOTT, P. A., 1975. Storage cycles in marine bivalve molluscs: A hypothesis concerning the relationship between glycogen metabolism and gametogenesis. Proc. 9th Europ. mar. biol. Symp., 191-211.

35 - HAMMEN, C. S., 1969. Metabolism of the oyster, *Crassostrea virginica*. Am. Zool., 9, 309-318.

36 - MUSTAFA, T. & HOCHACHKA, P. W., 1971. Properties of pyruvate kinases in tissues of a marine bivalve. J. Biol. Chem., 246, 3196-3203.

37 - SMYTH, J. D., 1976. Introduction to animal parasitology, 2nd. edition, Hodder and Stoughton, London, 466pp.

38 - BEUTLER, H-O., 1985. Succinate. In: Methods of Enzymatic Analysis. Vol VII Verlag Chemie, Basle. pp. 25-33.

39 - NOLL, F., 1984. Lactate. In: Methods of Enzymatic Analysis. Vol VI, Verlag Chemie, Basle. pp. 582-588.

40 - FISHER, W. S., 1988. Environmental influence on bivalve haemocyte function. American Fisheries Society Special Publication 18, 225-237.

41 - ALVAREZ, M. R. & FRIEDL, F. E., 1988. Factors affecting in vitro phagocytosis in haemocytes of the american oyster. Proc. PAMAQ III, Gloucester Point, Va., USA, 2-6 October 1988.

42 - FISHER, W. S. & NEWELL, R. I. E., 1986. Salinity effects on the activity of granular haemocytes of the american oyster *Crassostrea virginica*. Biol. Bull. 170, 122-134.

43 - AUFFRET, M., 1988. Bivalve haemocyte morphology. American Fisheries Society Special Publication 18, 169-177.

44 - CHAGOT, D. J., 1989. Caractérisation morphologique et fonctionelle des hémocytes *d'Ostrea edulis* et de *Crassostrea gigas*, mollusques bivalves. Études in vitro de leurs interactions avec le protozoaire *Bonamia ostreae* (Acetospora). E.P.H.E. thesis, Montpellier.

45 - WEISNER, B., 1984. Lysozyme (Muramidase). In: Methods of Enzymatic Analysis. Vol VI, Verlag Chemie, Basle. pp. 189-195.

46 - MEIATTINI, F., 1984. Inorganic peroxides. In: Methods of Enzymatic Analysis.Vol VII, Verlag Chemie, Basle. pp. 567-571.

47 - WIDDOWS, J. AND BAYNE, B.L., 1971. Temperature acclimation of *Mytilus edulis* with reference to its energy budget. J. mar. biol. Ass. U.K. 51, 827-843.

48 - BUXTON, C.D., NEWELL, R.C. AND FIELD, J.G., 1981. Response-surface analysis of the combined effects of exposure and acclimation temperatures on filtration, oxygen consumption and scope for growth in the oyster *Ostrea edulis*. Mar. Ecol. Prog. Ser. 6, 73-82.

49 - WEISER, W., 1973. Temperature relations of ectotherms: a speculative review. In: Effects of Temperature on Ectothermic Organisms. Ed. W. Weiser, Springer-Verlag, Heidelberg. pp. 1-23.

50 - MATHERS, N.F., 1974. Some comparative aspects of filter feeding in *Ostrea edulis* L. and *Crassostrea angulata* (Lam.) (Mollusca:Bivalvia). Proc. malac. Soc. Lond. 41, 89-97.

51 - DAVIDS, C., 1964. The influence of suspensions of micro-organisms of different concentrations on the pumping and retention of food by the mussel (*Mytilus edulis* L.). Neth. J. Sea Res., 2, 233-249.

52 - WINTER, J.E., 1973. The filtration rate of *Mytilus edulis* and its dependence on algal concentration, measured by a continuous automatic recording apparatus. Mar. Biol. 22, 317-328.

53 - SCHULTE, E. H., 1975. Influence of algal concentration and temperature on the filtration rate of *Mytilus edulis*. Mar. Biol. 30, 331-341.

54 - LOOSANOFF, V.L. AND ENGLE, J.B., 1947. Effects of different concentrations of micro-organisms on the feeding of oysters (O. virginica). Fishery Bull. Fish Wildl. Serv. U.S., 51, 31-57.

55 - LOOSANOFF, V.L., 1958. Some aspects of behaviour of oysters at different temperatures. Biol. Bull. mar. biol. Lab. Woods Hole, 114, 57-70.

56 - JORGENSEN, C.B., LARSEN, P.S. AND RIISGÅRD, H.U., 1990. Effects of temperature on the mussel pump. Mar. Ecol. Prog. Ser. 64, 89-97.

57 - THOMPSON, R.J. AND BAYNE, B.L., 1972. Active metabolism associated with feeding in the mussel *Mytilus edulis*. J. exp. mar. Biol. Ecol. 9, 111-124.

58 - BAYNE, B.L., 1973. Physiological changes in *Mytilus edulis* L. induced by temperature and nutritive stress. J. mar. biol. Ass. U.K. biol. Ass. 53, 39-58.

59 - NEWELL, R.C., JOHSON, L.G. AND KOFOED, L.H., 1977. Adjustment of the components of energy balance in response to temperature change in *Ostrea edulis*. Oecologia (Berl.) 30, 97-110.

60 - JORGENSEN, C.B., MOHLENBERG, F. & STEN-KNUDSEN, O., 1986. Nature of

the relation between ventilation and oxygen consumption in filter feeders. Marine Ecology Progress Series, 29, 73-88.

61 - FISHER, W.S., 1988. Disease Processes in Marine Bivalve Molluscs. American Fisheries Society Special Publication 18. 315 pp.

62 - BAYNE, B.L. AND THOMPSON, R.J., 1970. Some physiological consequences of keeping *Mytilus edulis* in the laboratory. Helgoländer wiss. Meeresunters.20, 526-552.

63 - AKBERALI, H.B. AND DAVENPORT, J., 1981. The responses of the bivalve Scrobicularia plana (da Costa) to gradual salinity changes. J. exp. mar. Biol. Ecol., 53, 251-259.

64 - PICKARD, G.L., 1979. Descriptive Physical Oceanography. Pergamom, Oxford. 233 pp.

65 - AKBERALI, H.B. AND DAVENPORT, J., 1982. The detection of salinity changes by the marine bivalve mollusc Scrobicularia plana (da Costa) and *Mytilus edulis* (L.) J. exp. mar. Biol. Ecol., 58, 59-79.

66 - DAVENPORT, J., 1979. The isolation response of mussels (*Mytilus edulis* L.) exposed to falling sea-water concentrations. J. mar. biol. Ass. U.K. 59, 123- 132.

67 - SHUMWAY, S.E., 1977. The effects of fluctuating salinity on the osmotic pressure and Na+, Ca2+, and Mg2+ concentrations in the haemolymph of bivalves. Mar. Biol. 41, 153-177.

68 - STICKLE, W.B. AND SABOURIN, T.D., 1979. Effects of salinity on the respiration and heart rate of the common mussel, *Mytilus edulis* L., and the black chiton, *Katherina tunicata* (Wood). J. exp. mar. Biol. Ecol. 41, 257-268.

69 - KINNE, O., 1971. Salinity: Animals. Invertebrates. In: Marine Ecology, Vol I, Environmental Factors, Part 2, ed. O. Kinne, Wiley-Interscience, New York. pp. 821-925.

70 - FINDLEY, A.M., BELISLE, B.W. AND STICKLE, W.B., 1978. Effects of salinity fluctuations on the respiration rate of the southern oyster drill Thais haemastoma and the blue crab Callinectes sapidus. Mar. Biol. 49, 59-67.

71 - PROSSER, C.L., 1973. Comparative Animal Physiology. Saunders, Philadelphia. 966 pp.

72 - SCHOFFENIELS, E., 1976. Adaptations with respect to salinity. Biochem. Soc. Symp. No. 41, 179-204.

73 - BAGINSKI, R.M. AND PIERCE, S.K., 1977. The time course of intracellular free amino acid accumulation in tissues of Modiolus demissus during high salinity adaptation. Comp. Biochem. Physiol. A 57A, 407-412.

74 - GREENWALT, D.E. AND BISHOP, S.H., 1980. Effect of aminotransferase inhibitors on the pattern of free amino acid accumulation in isolated hearts subjected to hyperosmotic stress. Physiol. Zool. 53, 262-269.

75 - ZURBURG, W. AND DE ZWAAN, A., 1981. The role of amino acids in anaerobiosis and osmoregulation in bivalves. J. exp. Zool. 215, 315-325.

76 - YANCEY, P.H., CLARK, M.E., HAND, S.C., BOWLUS, R.D., AND SOMERO, G.N. 1982. Living with water stress: Evolution of osmolyte systems. Science, 217, 1214-1222.

77 - BROOKS, S. E., 1989. Tidal stress effects on the defence mechanisms of Mercenaria mercenaria. BSc thesis, University of Southampton.

78 - BROOKS, J., 1990. The effect of tidally induced stress on the enzyme activity of the american hard shell clam Mercenaria mercenaria (L). BSc thesis, University of Southampton.

79 - FISHER, W. S. & CHINTALA, M. M., 1988. Summer stress on defence-related haemocyte activities of oysters, Crassostrea virginica. Proc. PAMAQ III, Gloucester Point, Va., USA, 2-6 October 1988.

80 - HUTCHINSON, S., HAWKINS, L. E., CRAWFORD, D. W. & LOCKWOOD, A. P. M., 1990. A study of thermal stress on the native oyster *Ostrea edulis*. CEGB final report. Fawley `B' Studies. Contract ref: C 14796. 94 pp.

81 - BALOUET, G. & PODER, M., 1979. A proposal for classification of normal and neoplastic types of blood cells in molluscs. In: Advances in Comparative Leukemia Research, eds D. S. Yohn, B. Lapin and J. Blakeslee. Elsevier, Amsterdam. pp. 205-208.

82 - CHENG, T. C., 1981. Bivalves. In: Invertebrate blood cells, Vol 2, eds N. A. Ratcliffe and A. F. Rowley, Academic Press, London. pp. 231-300.

83 - CALOW, P. & SIBLY, R. M., 1990. A physiological basis of population processes: ecotoxicological implications. Functional Ecology, 4, 283-288.

84 - HOLLOWAY, G. J., SIBLY, R. M. & POVEY, S. R., 1990. Evolution in toxin-stressed environments. Functional Ecology, 4, 289-294.

85 - HUTCHINSON, S. & HAWKINS, L. E., 1989. Stress assessment of *Ostrea edulis*. Porcupine, 4, 106-112.

86 - HUTCHINSON, S. & HAWKINS, L. E., 1992. Quantification of the physiological responses of the European flat oyster *Ostrea edulis* L. to temperature and salinity. J. Moll. Stud., 58, 215-226.

87 - KOEHN, R. K., 1984. The application of genetics to problems in the marine environment: future areas of research. Natural Environment Research Council discussion paper.

89 - FENG, S.Y., 1988. Cellular defence mechanism of oysters and mussels. Am. Fish. Soc. Spec. Publ., 18, 153-168.

90 - CHU, F.E., 1988. Humoral defence factors in marine bivalves. Am. Fish. Soc. Spec. Publ., 18, 178-188.

91 - BURTON. R.F., 1983. Ionic regulation and water balance. In The Mollusca : Physiology., (Chief Ed. K.M. Wilbur, Eds. : N.H. Verdonk, J.A.M. van der Biggelar, A.S. Tompa). 5 (2), 292-352. Academic Press

Inc., London.

92 - BARBER B.J., FORD S.E., HASKIN H.H., 1988. Effects of the parasite MSX (*Haplosporidium nelsoni*) on oyster (*Crassostrea virginica*) energy metabolism. I: Condition index and relative fecundity. J. Shellfish Res., 7: 25-13.

96 - FORD, S.E., 1986. Comparison of haemolymph proteins from resistant and susceptible oysters *C. virginica* exposed to the parasite *Haplosporidium nelsoni* (MSX). J. Invert. Path., 47, 283-294.

97 - MULVEY, M. and FENG, S.Y., 1981. Haemolymph constituents of normal and *Troctoeces maculatus* infected *Mytilus edulis*. Comp. Biochem. Physiol., 70A, 119-125.

100 - HAMMEN, C.S., 1968. Aminotransferase activities and amino acid excretion of bivalve molluscs and brachiopods. Comp. Biochem. Physiol., 26, 697-705.

102 - SONIAT, T.M. and KOENING, M.L., 1982. The effects of parasitism by *Perkinsus marinus* on the free amino acid composition of *Crassostrea virginica* mantle tissue. J. Shellfish Res., 2 (1), 25-28.

103 - MIALHE E., BOULO V., GRIZEL H., 1988. Bivalve mollusc cell culture. Amer. Fish. Soc.. Special Publication 18, 311-315.

106 - TRUSH M.A., WILSON M.E., VAN DYKE K., 1978. The generation of chemiluminescence (CL) by phagocytic cells. Methods. Enzymol., 57: 462-494.

107 - ALLEN R.C., 1986. Phagocytic leukocyte oxygenation activities and chemiluminescence : a kinetic approach to analysis. Meth. Enzymol., 133: 449-492.

108 - DIKKEBOOM R., TIJNAGEL J.M.G.H., MULDER E.C., VAN DER KNAAP W.P.W., 1987. Haemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. J. Inverteb. Pathol., 49: 321-331.

109 - BACHERE E., CHAGOT D., GRIZEL H., 1988. Separation of *Crassostrea gigas* haemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Dev. comp. Immunol., 12: 549-559.

111 - REMALEY A.T., KUHNS D.B., BASFORD R.E., GLEW R.H., KAPLAN S.S., 1984. Leishmanial phosphatase blocks neutrophil O_2^- production. J. biol. Chemis., 259: 11173-11175.

112 - HOLLINGDALE M.R., NARDIN E.H., THARAVANIJ S., SCHWARTZ A.L., NUSSENZWEIG R.S., 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells. An *in vitro* assay of protective antibodies. J. Immunol., 132: 909-913.

113 - RENWRANTZ L., MOHR W., 1978. Opsonizing effect of serum and albumin gland extracts on the elimination of human erythrocytes from the circulation of *Helix pomatia*. J. Inverteb. Pathol., 31: 164-170.

114 - SMINIA T., VAN DER KNAAP W.P.W., ELLENBOSCH, 1979. The role of serum factors in phagocytosis of foreign particles by blood cells of the fresh water snail *lymnaea stagnalis*. Dev. comp. Immunol., 3: 37-44.

115 - KLEBANOFF S.J., CLARK R.A., 1978. The neutrophil: function and clinical disorders. North-Holland publishing company, pp. 810.

116 - BABIOR B.M., 1984. Oxidants from phagocytes: agents of defence and destruction. Blood, 64: 959-966.

117 - MAUEL J., 1984. Mechanisms of survival of protozoan parasites in mononuclear phagocytes. Parasitology, 88: 579-592.

118 - HERVIO D., CHAGOT D., GODIN P., GRIZEL H., MIALHE E., 1991. Localization and characterization of acid phosphatase activity in *Bonamia ostreae* (Ascetospora), an intrahaemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia). Dis. Aquat. Org., 12:67-70.

119 - CHANG C.S., CHANG K.P., 1986. Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of *Leishmania*-macrophage binding. Proc. Natl. Sci., USA, 83: 100-104.

120 - WIRTH J.J., KIERSZENBAUM F., 1987. Inhibition of mammalian host cell infection by insect-derived, metacyclic forms of *Trypanosoma cruzi* in the presence of human or rabbit anti-*T. cruzi* antibodies. Int. J. Parasitol., 17: 841-845.

121 - FROMTLING R.A., RUZZO R., 1985. Chemiluminescence as a tool for the evaluation of antimicrobial agents: a review. Meth. Find. Expt. Clin. Pharm., 7: 493-500

122 - BACHERE E., NOEL T. AND MIALHE E., 1991. Effects of environmental pollutants and chemotherapeutic agents on the chemiluminescence of the haemocytes from the oyster *Crassostrea gigas*. EAS, Dublin, 10-12 june 91.

123 - MOR N., GOREN M.B., PABST M.J., 1988. *Mycobacterium lepraemurium* activates macrophages but fails to trigger release of superoxide anion. J. Immunol., 140: 3956-3961.

124 - MCCABE R.E., MULLINS B.T., 1990. Failure of *Trypanosoma cruzi* to trigger the respiratory burst of activated macrophages. Mechanism for immune evasion and importance of oxygen-independent killing. J. Immunol., 144:2384-2388.

125 - EL-ON J., ZVILLICH M., SAROV I., 1990. *Leishmania major*: inhibition of the chemiluminescent response of human polymorphonuclear leukocytes by promastigotes and their excreted factors. Parasite Immunology, 12: 285-295.

126 - VINCENDEAU P., DAULOUEDE S., 1991. Macrophage cytostatic effect on *Trypanosoma musculi* involves an L-arginine-dependent mechanism. J. Immunol., 146: 4338-4343.

127 - VAN BANNING, P., 1988. Management strategies to control diseases in the dutch culture of edible oysters. Am. Fish. Soc. Spec. Publ., 18, 243-240.

128 - BOUGRIER, S., TIGE, G., BACHERE, E. and GRIZEL, H., 1986. *Ostrea angasi* acclimatization to French coasts. Aquaculture, 58, 151-154.

129 - LE BEC, C., MAZURIE, J., COHENNEC, N. and LE GOGUIC, Y., 1991. Influence of *Crassostrea gigas* mixed with *Ostrea edulis* on the incidence of *Bonamia* disease. Aquaculture, 93,(3), 263-272.

130 -PARKS D.R., BRYAN V.M., HERZENBERG L.A., 1979. Antigen specific identification and cloning of hybridomas with a fluorescence-activated cell sorter (FACS). Proc. Natl. Acad. Sci. USA, 76: 1962.

131 - REED J.L., MUENCH H., 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg., 27: 493-497.

132 - CULLOTY S.C., MULCAHY M.F., 1990. An evaluation of anaesthetics for *Ostrea edulis* L.. 4th internat. Colloq. marine Aquacul., 17-21 Sept., Vigo (Pontevedra), Spain.

133 - SINDERMANN, C.J., 1990. Principal diseases of marine fish and shellfish Vol. 2 Diseases of marine shellfish. Academic Press, London, 516 pp.

134 - FORD, S.E., 1988. Host-parasite interactions in Eastern oysters selected for resistance to *Haplosporidium nelsoni* (MSX) disease : survival mechanisms against a natural pathogen. Am. Fish. Soc. Spec. Publ., 18, 206-224.

135 - NEEDLER, A.W.H. and LOGIE, R.R., 1947. serious mortalities in Prince Edward Island oysters caused by contagious disease. Trans. Royal Soc. Can., 41, 73-89.

136 - FORD S., HASKIN H.H., 1987. Infection and mortality patterns in strains of oysters *Crassostrea virginica* selected for resistance to the parasite *Haplosporidium nelsoni* (MSX). J. Parasit., 73: 368-376.

137 - FORD S.E., FIGUERAS A., RUTGERS A. J., 1986. Effects of MSX (*Haplosporidium nelsoni*) parasitism on reproduction of the oyster, *Crassostrea virginica*. Nat. Shellfisheries Assoc., 78th annual NSA meeting, Seattle, Washington, June 22-26, 55.

137 - LONGWELL, A.C. and STILES, S.S., 1973. Oyster genetics and the probable future rôle of genetics in Aquaculture. Malacol. Rev., 6, 151-177.

138 - HASKIN, H.H. and FORD, S.E., 1987. Breeding for disease resistance in molluscs. Proc. World Symp. on Selection, Hybridisation and Genetic Engineering, Bordeaux 27-30 May 1986, 2, 431-441.

139 - PODER M., CAHOUR A., BALOUET G., 1982. Haemocytic parasitosis in European oyster *Ostrea edulis* L.: pathology and contamination. Proceeding of the 15th annual meeting of the Society for the Invertebrate Pathology, Brighton, England, 254-257.

140 - ELSTON R.A., KENT M., WILKINSON M., 1987. Resistance of *Ostrea edulis* to *Bonamia ostreae* infection. Aquaculture, 64: 237-242.

141 - FORD S.E., HASKIN H.H., 1988. Management strategies for MSX (*Haplosporidium nelsoni*) disease in eastern oysters. Amer. Fish. Soc.. Special Publication 18, 249-256.

142 - DINAMANI P., HINE P.M., JONES J.B., 1987. Occurence and characteristics of Bonamia ostreae, in the New-Zealand dredge oyster, Tiostrea lutaria. Dis. aqu. Org., 3: 37-44.

ANNEXES

ANNEXE 1

ADENYLATES AND ADENYLATE ENNERGY CHARGE (AEC)
Preparation of reagents

1) 0.5 M triethanolamine hydrochloride (TEA) - 93 g dissolved in 400 cm^3 of water and adjusted to pH 7.6 with NaOH, then made up to 1 litre with water. This stock solution was kept at 2-4°C and was diluted x 10 to 0.05M before use.

2) 0.5 M MgCl2.6H20 - 10.17 g dissolved in water and stored at 2-4°C.

3) 0.5 M MgSO4.7H20 - 12.32 g dissolved in water, made up to 100 cm3 and stored at 2-4°C.

4) 2 M KCl - 29.82 g dissolved in water, made up to 200 cm3 and stored at 2-4°C.

5) Ethylenediaminetetracetic acid (EDTA) - a saturated solution made by suspending 5 g in 50 cm^3 water.

6) 5% w/v $NaHCO_3$ in water.

7) 10 mM nicotinamide dinucleotide, reduced sodium salt (NADH-Na_2) - made up from a solution of 7.09 g cm^{-3} in solution 6 and divided into 0.75 cm^3 aliquots in foil-covered vials since this compound is light sensitive. Stored at 20°C.

8) 20 mM NADP+ - aqueous solution of 15.75 mg cm^{-3} divided into 0.75 cm^3 aliquots in foil-covered vials since this compound is light sensitive. Stored at -20°C.

9) 0.4 M D-glucose - 7.21 g dissolved in 100 cm^3 of water, divided into 1 cm^3 aliquots. Stored at -20°C.

10) 40 mM phosphoenolpyruvate, monosodium salt (PEP) - 9.44 mg cm-^{3} aqueous solution divided into 1.0 cm^3 aliquots and stored at -20°C.

11) 10 mM ATPNa2H_2 - aqueous solution of 6.05 mg cm^{-3} divided into 0.25 cm^3 aliquots. Stored at -20°C.

12) 20 mM ADP-Na_2 - aqueous solution of 9.42 mg cm^{-3} divided into 0.1 cm^3 aliquots and stored at -20°C.

13) 20 mM AMP-$Na_2.6H_2O$ - aqueous solution of 9.98 mg cm^{-3} divided into 0.5 cm^3 aliquots and stored at -20°C.

14) 3.2 M $(NH_4)2SO_4$ - aqueous of 211.42 g in 500 cm^3 water stored at roomtemperature.

15) 0.6 mg cm^{-3} G6PDH (from yeast, Grade II) - 5 mg cm^{-3} suspension in 3.2M $(NH_4)2SO_4$. 60 µl of stock enzyme plus 440 µl of solution 14 was used. Stored at 2-4°C.

16) 2.0 mg cm^{-3} HK (from yeast, Grade II) - 10 mg cm^{-3} suspension in 3.2 M $(NH_4)2SO_4$. 200 µl of enzyme added to 800 µl of solution 14. Stored at 2-4°C.

17) 1.0 mg cm^{-3} LDH (from rabbit muscle) - 10 mg cm^{-3} suspension in 3.2 M $(NH_4)2SO_4$. 60 µl of stock enzyme added to 240 µl of solution 14. Stored at 2-4°C.

18) 2.0 mg cm^{-3} PK (from rabbit muscle) - 10 mg cm^{-3} suspension in 3.2 M $(NH_4)2SO_4$. 60 µl of stock enzyme added to 240 µl of solution 14. Stored at 2-4°C.

19) 1.25 mg cm^{-3} MK (from rabbit muscle) - 5 mg cm^{-3} suspension in 3.2 M $(NH_4)2SO_4$. 75 µl of stock enzyme added to 225 µl of solution 14. Stored at 2-4°C.

ATP assay buffer

Reagent	cm^3
TEA	71.25
MgCl	0.75
NADP	0.75

ADP-AMP assay buffer

Reagent	cm3
TEA	65.00
MgSO4	0.88
KCl	1.88
EDTA	0.125
NADH	0.75
PEP	1.00
ATP	0.25

Standard Mixture

0.05 mM ATP + ADP + AMP: Take 0.1 cm^3 of each nucleotide, ATP, ADP and AMP, added to 3.7 cm^3 water, mixed, placed on ice. Made up on the day of use. 0.1 cm^3 of the standard mixture was placed in a cuvette with an assay volume of 3.0 cm^3 to give the following changes in absorbance at 340 nm: ΔA_{ATP}: 0.096 ± 0.003 (S.E.): ΔA_{ADP}: 0.081 ± 0.005; ΔA_{AMP}: 0.194 ± 0.003.

ANNEXE 1 bis

Calculation of results

Nucleotide Concentrations: The concentrations of ATP, ADP and AMP were calculated as follows:

$$ATP\ (\mu mol\ g^{-1}\ wet\ wt\ tissue) = \frac{\Delta A_{ATP} \times AV \times EV}{6.22 \times SV \times TW \times 1.0}$$

$$ADP\ (\mu mol\ g^{-1}\ wet\ wt\ tissue) = \frac{\Delta A_{ADP} \times AV \times EV}{6.22 \times SV \times TW \times 1.0}$$

$$AMP\ (\mu mol\ g^{-1}\ wet\ wt\ tissue) = \frac{\Delta A_{AMP} \times AV \times EV}{6.22 \times SV \times TW \times 1.0}$$

where:

ΔA_{ATP} = A2 - A1

ΔA_{ADP} = B1 - B2 If $\Delta A < 0$, use 0,

ΔA_{AMP} = B2 - B3 not the negative number.

AV = assay volume (cm^3), i.e. volume of solution (buffer and sample) in cuvette.

EV = extract volume (cm^3), i.e. volume of neutralized supernatant measured after extraction procedure.

6.22 = extinction coefficient, i.e. absorbency at 340 nm and pH 7.6 of a solution of NADPH or NADH

SV = volume of sample supernatant used in cuvette (cm^3).

TW = tissue weight (g).

1.0 = length of light path inside cuvette (cm); usually 1.0 cm.

ANNEXE 2

SUCCINATE MEASUREMENTS
Preparation of reagents

All solutions were made with distilled water and the containers were sterilized to prevent microbial contamination.

1. Glycylglycine buffer (glycylglycine, 0.30 M, pH 8.4; Mg^{2+}, 41 mM): 2.4g glycylglycine and 600 mg $MgSO_4.7H2O$ dissolved in ca.50 cm^3 water, adjusted to pH 8.4 with sodium hydroxide, 2 M, and made up to 60 cm^3 with water.

2. Nicotinamide-adenine dinucleotide, reduced (ß-NADH, 9 mM): (23) mg NADH, disodium salt, and 30 mg $NaHCO^3$ dissolved in 3.0 cm^3 water.

3. Coenzyme A/inosine 5'-triphosphate/phosphoenolpyruvate solution (CoA, 11 mM; ITP, 15 mM; PEP, 21 mM): 30 mg CoA, trilithium salt, 30 mg ITP, disodium salt, and 30 mg PEP, tricyclohexylammonium salt, dissolved in 3 cm^3 water.

4. Enzyme mixture (PK, 600 kUl-1 ; LDH, 550 kUl^{-1}): diluted stock suspensions (PK from rabbit muscle, suspended in ammonium sulphate solution, 3.2 M, pH 6.0, ≥200 Umg^{-1} protein, at 25°C, PEP as substrate; LDH from rabbit muscle, suspended in ammonium sulphate solution, 3.2 M, pH 7.0, ≥550 Umg^{-1} protein, at 25°C, pyruvate as substrate) with ammonium sulphate solution 3.2 M.

5. Succinyl-CoA synthetase (50 kUl^{-1}): stock suspension of succinate thiokinase (from pig heart, suspended in ammonium sulphate solution, 3,2 M, pH 6.0, ≥10 Umg^{-1} protein, at 25°C, succinate as substrate) undiluted.

6. Perchloric acid solution (0.8 M): 6.70 cm^3 perchloric acid, sp. gr. 1.67, 70% w/w, with 40 cm^3 ethanol, 95% v/v, and made up with water to 100 cm^3.

7. Perchloric acid solution (0.6 M): 5.0 cm^3 perchloric acid, sp. gr. 1.67, 70% w/w, with 40 cm3 ethanol, 95% v/v, made up with water to 100 cm^3.

8. Triethanolamine/K2C03 solution (TEA, 0.2 M; K_2CO_3, 2.3 M): 1.0 g TEA hydrochloride and 8.0 g K_2CO_3 with ca. 20 cm^3 water. pH adjusted to 8.2 with KOH, 2 M, and made up with water to 25 cm^3.

Stability of solutions: all solutions were stored in a refrigerator at 0°C to 4°C. Solution (1) is stable for 4 weeks if growth of micro-organisms is avoided. The NADH solution (2) is stable for 4 weeks. The coenzyme solution (3) can be stored for 2 weeks, and the suspensions (4) and (5) as well as solutions (6)-(8) are stable indefinitely if microbial contamination is avoided.

ANNEXE 3

LACTATE MEASUREMENTS
Preparation of reagents

all solutions were made with distilled water.

1. Neutral L-(+)- glutamate solution (0.77 M): 56.75 g L-(+)-glutamic acid dissolved in 380 cm^3 NaOH, 1 M and made up to 500 cm^3 with water.

2. Glutamate buffer (0.52 M, pH 8.9): 20 cm^3 neutral glutamate solution (1) mixed with 9.4 cm^3 NaOH, 1 M.*

* The addition of this large amount of NaOH was necessary because NH_4^+ present in relatively high amounts in the enzyme preparations (LDH and ALT) is partly undissociated at pH 8.9 and therefore free H^+ ions arise.

3. Perchloric acid (0.6 mol l^{-1}): 10.4 cm^3 $HClO_4$ (sp. gr. 1.67, 70% w/w) diluted to 200 cm^3 with water.

4. Nicotinamide-adenine dinucleotide (ß-NAD, 24 mM): 75 mg NAD, free acid, in water and made up to 4 cm^3.

5. Methylene orange indicator (0.05%, w/v): 25 mg methylene orange in 50 cm^3 dissolved in water.

6. Lactate dehydrogenase (LDH, 550 kU l^{-1}): stock suspension (LDH from skeletal muscle, suspended in ammonium sulphate solution, 3.2 M, ≥ 200 U mg^{-1} protein, at 25°C) mixed with ammonium sulphate solution, 3.2 M.

7. Alanine aminotransferase, ALT (80 kUl^{-1}): stock suspension (ALT from liver, suspended in ammonium sulphate solution, 1.8 M, ≥ 30 U mg^{-1} protein at 25°C) mixed with ammonium sulphate solution, 1.8 M.

Stability of solutions: solutions (2), (3), (6) and (7) were stored stoppered in a refrigerator at 0°C to 4°C. Solutions (1) and (4) were stored frozen at -25°C. The LDH suspension (6) is stable for months; however, the ALT suspension (7) loses some activity after 10 weeks. Fresh NAD solution (4) were prepared every 2 weeks. The neutral glutamate solution (1) is stable indefinitely if growth of micro-organisms is avoided. Solution (2) can be stored for 2 weeks.

ANNEXE 4

HYDROGEN PEROXIDE CONCENTRATION MEASUREMENTS
Preparation of reagents

6% perchloric acid - 10 cm^3 of 60% perchloric acid made up to 100 cm^3 with distilled water, in a volumetric flask.

6 M sodium hydroxide solution - 24 g of NaOH in 70 cm^3 of distilled water, and then made up to 100 cm^3.

Hydrogen peroxide standard solution - 1.628 cm^3 of stock H_2O_2 into a volumetric flask, and made up to 100 cm^3 with distilled water. This gave a H_2O_2 solution of 20 mg l^{-1}

Peroxide reagent - 2.67 g Na_2HPO_4, 1.726 g $NaH_2PO_4.H_2O$, 0.1 g NaN_3, 100 µl Triton X-100, 0.004 g POD, 0.01 g 4 aminophenazone, 0.4 g chromotropic acid $Na_2.2H_2O$, dissolved in 100 cm^3 of distilled water. The azide was present to prevent bacterial action and inhibit the action of catalase (which may be present in biological samples), and the Triton X-100 helped to solubilise any lipid fractions. The reagent was kept in a stoppered, brown bottle at 0-4°C.

The peroxide reagent was made up once a month. The hydrogen peroxide standard solution was freshly prepared for each assay. The perchloric acid and sodium hydroxide solution are both stable indefinitely at room temperature.

ANNEXE 5

LYSOZYME CONCENTRATION MEASUREMENTS

Assay mixture - 0.006 g of *Micrococcus luteus* lyophilisate suspended in 30 cm^3 of phosphate buffer, to give a concentration of 0.2 g l^{-1}.

Phosphate buffer - 8.05 g NaH_2PO_4, 2.6 g Na_2HPO_4, 0.9 g NaCl, 0.52 g NaN_3, dissolved in 500 cm^3 of distilled water. This was adjusted to pH 6.3 with 0.1M HCl, and then diluted to 1000 cm^3 with distilled water.

The phosphate buffer was stored in a cupboard at room temperature. The Micrococcus suspension was made up fresh for each assay and maintained at 25°C in a water bath up to its point of use.

The method was calibrated using lysozyme standards:

1. Lysozyme stock solution (1 mg cm^{-3}, 40100 units cm^{-3}): 2 mg human lysozyme in 2 cm^3 water.

2. Lysozyme standard solutions:

a) 0.1 cm^3 solution (1) with 2.9 cm^3 NaCl, 0.0154 mol l^{-1};

b) 0.5 cm^3 solution (2a) with 0.5 cm^3 water (668 k units l^{-1});

c) 0.5 cm^3 solution (2a) with 1.5 cm^3 water (334 k units l^{-1});

d) 0.25 cm^3 solution (2a) with 1.75 cm^3 water (167 k units l^{-1});

e) 0.25 cm3 solution (2a) with 3.75 cm3 water (83.5 k units l^{-1});

f) 0.25 cm3 solution (2a) with 7.75 cm3 water (42 k units l^{-1});

Stability of solutions: solution (1) can be kept for up to 6 days at +4°C, and for several weeks at -20°C. Solutions (2a)-(2f) are stable for 4 hours at room temperature.

E. C. D. G. XIV PROJECT 1-272

WORKING SESSION ON BONAMIASIS

IFREMER La Tremblade

21 January - 1 March 1991

GENERAL ORGANIZATION:

Monday (M)	Protocol establishment
Thuesday (T) *Wednesday* (W) *Thursday* (T)	Experimentations
Friday (F)	Results analysis and discussion.

FIRST WEEK (21 - 25 January).

M - Presentation of theoritical aspects and results already obtained on:

1. Parasite purification, experimental pathology.
2. Hemocyte primary-cultures, *in vitro* infections with *B. ostreae*, interactions pathogen/hemocytes.
3. Chemiluminescence, Respiratory Burst, interactions of *B. ostreae* with the hemocyte Respiratory Burst.

T - Presentation of the working programme for 6 weeks.

W.T.F. - Practical initiation to the main techniques:

1. *B. ostreae* purification and experimental infections of *O. edulis* and *C. gigas*. Determination of the 50% infectious dose.
2. Hemocyte *in vitro* culture for viability in relation to time, hemogram establishment.
3. Chemiluminescence for *O. edulis* and *C. gigas*, study of individual variability of CL responses. Zymosan stimulation (Zy).

SECOND WEEK (28 Jan - 1 Feb).

T.W.T. - Experimental infections of *O. edulis* with *B. ostreae* Comparison of two infectious doses.
Analysis of the early stages of infections (before two months): detection of parasites by histology on cytocentrifuged preparations or by PCR (enzymatic amplification of DNA).

- Comparative analyses of the hemograms and of the chemiluminescent responses of two "strains" of *O. edulis* (one standard from Mediterranean and one out on resistance selection).

THIRD WEEK (4 - 8 February).

From this week, three groups will work separately and each day on a different experimentation.

T - *In vitro* infections of *O. edulis* and *C. gigas* with *B. ostreae (Bo).*
1. Parasite purification
2. Hemocyte primary-cultures and infection with *Bo.* Cytospin preparations at different times: 2, 12, 24 and 48h
3. Hemogram determination (observation of the slides prepared on week 1).

W
1. Chemiluminescence with P.M.A. (10 animals for *O. edulis* and *C. gigas)*
2. Hemogram determination (week 1)
3. Observation of cytospin preparations (2 h, 12 h).

T
1. Chemiluminescence assays with L.P.S. as activator, Zy stimulation (10 animals per species)
2. Hemogram determination (week 1)
3. Observation of cytospin preparations (24 h, 48 h).

FOURTH WEEK (11 - 15 February).

In vitro infections of *O. edulis* and *C. gigas* with *B. ostreae (Bo).*

T - *In vitro* infections / opsonization with oyster serum.
1. Parasite purification
2. Hemocyte primary-cultures / *Bo* opsonization and hemocyte infection
3. Chemiluminescence / *Bo* opsonized - Zy opsonized.

W - *In vitro* infections / monoclonal antibodies (MAB) specific of *Bo.*
1. Purification
2. Hemocyte primary-cultures / *Bo* treatment with MAB and hemocyte infection
3. Chemiluminescence / *Bo* MAB - Zy MAB.

T - *In vitro* infections / Protease inhibitors (PMSF, TLCK).
1. Purification
2. Hemocyte primary-cultures / *Bo* treatment with PMSF or TLCK / infection
3. Chemiluminescence / Bo PMSF or TLCK - Zy PMSF or TLCK.

FIFTH WEEK (18 - 22 February).

Chemiluminescence on *O. edulis* and *C. gigas* hemocytes.

T - Separated cell types: granulocytes, hyalinocytes and total hemolymph
- Two species
- Stimulation with Zy and PMA.

W - Cells treated with MAB specific of *C. gigas* hemocytes
- *C. gigas*
- Stimulation with Zy.

T - Cells treated with MAB specific of *C. gigas* hemocytes
- *O. edulis*
- Stimulation with Zy.

SIXTH WEEK (25 Feb - 1 March).

Results analysis
Discussion....