Commission of the European Communities

Contract No. FAIR 95-421

Genetic bases and variability of physiological traits involved in growth in *Crassostrea gigas*

SECOND PROGRESS REPORT
1st January-31st December 1997

Contractors:

1. Institut Francais pour la Recherche et l'exploitation de la Mer, (IFREMER), France
2. Plymouth Marine Laboratory, Plymouth, United Kingdom
3. Observatoire Océanologique de Villefranche/Mer, Villefranche sur mer, France
4. University College of Galway, Galway, Ireland
5. Institute of Marine Biology of Creete, Iraklion, Greece
6. Laboratoire Génome et Population, Montpellier, France

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"GENEPHYS"

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1st January-31st December 1997
FAIR 95-421

ANNEX I
Abstract

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Abstract of the Progress Report for the period from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

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EC contact : DG XIV / C.2

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Partner 4	University College of Galway **(UCG)**	Contractor	Dr. N. P. WILKINS
Partner 5	Institute of Marine Biology of Creete **(IMBC)**	Contractor	Dr. E. ZOUROS
Partner 6	Laboratoire Génome et Population **(LGP)** CNRS-URA-1493	Ass. Contractor to the coordinator	Dr. F. BONHOMME

Abstract

1. *Objectives*

The UE project "GENEPHYS", initiated by the IFREMER laboratory GAP-La Tremblade (Génétique Aquaculture et Pathologie) and CREMA-L'Houmeau (Centre de Recherche en Ecologie marine et Aquaculture), aims to establish the relationships between physiological traits involved in growth (oxygen consumption, absorption efficiency, scope for growth) and their genetic bases (determinism, variability within and among populations) in cupped oyster *Crassostrea gigas*.

The main objectives are :

1. To express the maximum variability of growth, both at the larval stage and at the adult stage, in a oyster population originating from parents of various origins.

2. To check the persistence over the time, of growth performance and related physiological functions.

3. To explain the observed growth differential by physiological functions studied.

4. To establish whether the growth differential by physiological functions are associated with a genetic basis, using 2 types of markers (allozymes and microsatellites), and whether these are related to aneuploidy and the identity of missing chromosomes.

5. To estimate, in a second generation, genetic variance (additivity, dominance) and heritability of growth and of related physiological traits. Also to estimate aneuploidy transmission to this generation.

6. To evaluate the possibility of selection for a better assimilation efficiency. The beginning of a genetic map provides the first step for marker-assisted selection and QTL (Quantitative Trait Loci) location.

2. *Description of work*

Intra-population crosses were carried out in 1996 and led to 3 populations which were reared under non selective conditions in the La Tremblade hatchery.

These populations were first sampled for microsatellites and allozymes analyses at the juvenile (6 months old) stage.

Individual growth was then recorded from early stage in La Tremblade hatchery. A sample of these oysters were then used for the determination of the physiological parameters followed by the measurement of the protein turn-over. A the end of this trial, animals were dissected and shared for stress protein and digestive enzymes assays,

allozyme and microsatellite analyses. Another sample was used to evaluate if the physiological parameters were stable over the time. For this purpose, 30 adult oysters have been currently recorded (5 times in total) in the year using a continuous monitoring system.

A third set of these oysters was transferred to the Bouin hatchery for growth recording under 3 levels of competition for food. At the end of the monitoring period, some of these adult oysters were sampled for allozyme and microsatellite genotyping, aneuploidy level and for stress protein assays. New methods of chromosome labelling (G and R-banding and FISH) were initiated. G and R-bands permitted a complete identification of chromosomes.

3. State of progress

The present 5-year project has now reached its second year. The first year was mainly dedicated to the development of biological material, of new techniques to measure the physiology of oysters and new genetic markers. This second year has yielded a large amount of data from the different partners. Growth, physiology and genetics have been investigated on common material with the same individual oysters being shared among the partners.

Production and management of the G1and G2 generations

This task was performed in 1996 and produced 3 experimental populations from crosses of 5 males and 5 females originating from 3 natural populations. The production and management of the G2 generation will be performed in 1998.

Recording of the growth performances

A first group out of the oysters from the 3 5X5 crosses performed in 1996 were individually labelled.. One of the objectives was to characterise the growth of oysters in order to study their physiological performance. A large range of growth performances were observed at an early stage and 100 oysters were chosen for physiological studies. The other main topic to be assessed this year was the maintenance of growth performance over years. Two experiments were carried out at Bouin, La Tremblade and Palavas in order to address this question.

The breeding and the rearing of the oysters was designed to generate a population with a high variability in growth. Overall, growth rate in *Crassotrea gigas* appears to be fairly well maintained over time, under laboratory conditions. About 50 % of the variation observed in year 2 can be explained by the weight reached at the end of year 1.

The experiment in Bouin, where the 3 5X5 populations were reared under different levels of competition for food (high, medium and low), provides new results on variability and plasticity for growth. Oysters reared under low and medium levels of competition for food showed similar growth performances. There is an absence of interaction between competition level (i.e. tank) and the relationship between initial total

weight and slope of growth. Growth recordings on the oysters reared in Palavas are still in progress, the animals will be weighed early in 1998.

Growth rate is significantly different between 5X5 crosses however. This would tend to support the idea of a genetic basis to the difference in growth between populations.

Physiological analysis

According to the last annual report two main experiments were performed this year : temporal stability of physiological traits and characterisation of physiological performance (physiological functions, protein turn-over, proteolytic and enzyme activity) of animals from the G1 generation.

Continuous monitoring systems were developed to measure the acquisition of energy (filtration) and its loss, measured by the metabolism (oxygen consumption). This was performed in order to improve the precision of the estimation of physiological parameters (mainly the scope for growth). Such a system was developed in 1989 for respiration and more recently (September 1997) for filtration.

Experiments were performed in April, September, October and December. The observed mean value of RTA (Respiratory Time Activity: percentage of time the animal spent consuming oxygen relative to the experimental time) was in complete agreement with previous experiments. At present, it could be concluded that there is no temporal stability, but as it was the first time that such measurements (rather than estimations) were made, it is difficult to explain the observed differences. The complete treatment of the five planned experiments should give more information about time activities and physiological behaviour of oysters. This will allow us to test the temporal stability of physiological traits definitively.

The same G1 animals, acclimated to temperature and low food availability, were analysed for non-destructive measurements of the physiological functions, protein turn-over estimation, proteolitic activity determination and digestive enzyme activity measurements. Results showed that, except for dry weight, there is no significant differences between populations for feeding processes and for digestive enzymes.

Differences observed for digestive enzymes could be explained by the existence of two sets of animals. Oysters from the first set showed lower enzyme activities, characterised by a larger affinity to ingest food than the oysters of the second set. Similarly, two groups of animals could be defined according to their relationships between scope for growth (SFG) and total weight. Inside the first group, the large SFG values were observed in larger oysters, while low SFG were measured in smaller animals. Inside the second group in contrast, high SFG values, even null or negative, were observed in small oysters, while high negative SFG were observed in large oysters.

Genetic analyses

The use of 4 highly polymorphic microsatellite loci has been standardised, allowing a

safe and technically easy means of identifying parents of the progeny in mixed or mass crosses. This already accomplishes one of the main objectives of the microsatellite analysis in the project. Around 90% of parentage can be determined using only two loci.

In our crosses, the contribution of male and female parents (G0) to the next generation (G1) is highly unbalanced, as revealed by the microsatellite analysis. Some animals do not contribute to the next generation at all, whilst others contribute the majority of the progeny.

The inheritance between the parents and the offspring of the 3 "5x5" crosses were also examined using allozyme markers. Strong deviations from Mendelian expectations were observed at various loci in the 3 populations. These deviations can be directly related to the unbalanced contributions of the parental G0 oysters in each cross. No correlation was detected between heterozygosity at allozyme loci and growth (using total weight) in the three populations.

A MgCl2 treatment was tested on individually labelled oysters in order to score aneuploidy on biopsies of gills. The mitosis were absent or scarce in slide preparations made from animals from the three 5 x 5 populations. As no non-destructive method could be developed to select broodstocks with a known aneuploidy, the study of a possible transmission of aneuploidy in the next offspring will be done by a destructive method, i.e. immediately after spawning.

The comparison between the percentage of aneuploidy in ten small animals from each population and in ten large animals confirms the correlation between somatic aneuploidy and growth rate. Looking at all animals studied, the total percentages of aneuploidy in the populations are slightly lower than those observed last year in the pseudo-cohorts, but still remains relatively high.

The search for chromosome markers is based on two different kinds of techniques : chromosome banding (G and R-bands) and FISH . G-bands allowed us to identify the different pairs of a karyotype which showed a specific pattern. "R-bands" permit a complementary diagnostic for identification of chromosomes. These results are the first on G and R-bands obtained in oysters.

During this second reporting period, a large cosmid based genomic DNA library has been constructed. This cloning of *G. gigas* genomic DNA is now complete. The main research emphasis of the second reporting period was the development of methods to prepare suitable chromosome spreads for *C. gigas*. The method has been optimised and the *C. gigas* chromosome spreads obtained appear suitable for fluorescent in situ hybridisation analysis using DNA probes.

4. Achievements

Growth, physiology and genetics have been investigated on common material with the same individual oysters being shared among the partners. The actions planned in 1996

for 1997 were achieved, even if all the results are not yet fully treated. Nevertheless the progress of the project shows many new and interesting results, bringing new insight on growth in oysters.

Growth recording on oysters in the experiments run in La Tremblade, Bouin (and data expected from Palavas) demonstrate that growth performance is well maintained over time from the first to the second year and that variability is very large in our biological material. The estimation of individual growth performances of 1500 labelled oysters allows us to set up the basis of a selection experiment for growth.

Genetic analyses of our 3 experimental populations gives, for the first time, results for both allozymes and microsatellite markers on the same biological material. The joint analysis has not been done but will be performed in 1998.

For the physiological analyses, it could be concluded at present that there is no temporal stability of physiological traits. More data are needed however to explain the differences observed over time. The results of the June experiment, performed on G1 oysters acclimated to temperature and low food availability, showed two different groups of behaviour both for digestive enzymes and scope for growth. A surprising linear relationship between dry weight and oxygen consumption was noticed in the June experiment.

5. Future actions

Genetical and physiological analysis on the G1 generation which will be completed in 1998 :

- growth performances recorded in Palavas,
- allozymes and microsatellites on adults,
- microsatellite analysis on larval populations,
- temporal stability of physiological traits,
- analysis of protein turn-over and proteolitic activities.

The global treatment of all the data collected by the different partners in 1997 and 1998 will be done and should provide new results of high scientific value on the genetics and physiology of growth in C. gigas. The comparison and the combined analysis of the results from each partner will be of great interest as different methods have been applied on the same biological material.

1998 will be mainly dedicated to the production of the G2 generations, based on physiological performances, aneuploidy level and growth of the selected parents. To estimate genetic parameters such as heritability for growth, a new G1 generation, consisting this time of full-sib families, will be also initiated.

European Commission

Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Consolidated Progress Report
1st january-31st december 1997

Reporting Period:
1st January-31st December 1997
FAIR 95-421

ANNEX II
Consalidated Progress Report
Title page

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Consolidated Progress Report for the period
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FAIR PL. 95.421

Scientific Synthesis

1. Introduction

The present 5-year project has now reached its second year. The first year was mainly dedicated to the development of :

- biological material (i.e. 3 experimental populations of *C. gigas* produced under controlled hatchery conditions)
- new techniques to measure the physiology of oysters (continuous monitoring, stress proteins and proteolytic enzymes)
- new genetic markers (microsatellites).

Due to some problems during the early rearing of the juvenile oysters in the first year, a change in biological material was decided upon at the first-year meeting. However, the main objectives of the project remained unchanged and the first-year report of this project was accepted by the Commission.

This second year has yielded a large amount of data from the different partners. Growth, physiology and genetics have been investigated on common material with the same individual oysters being "shared" (scientifically and also literally) among the partners. This report presents results which were, in some cases, only obtained at the end of 1997. The full analysis of these data will therefore require more time and completion of some of the experiments is also still in progress. Here we summarise the present progress of the GENEPHYS program and its forthcoming perspectives.

2. Results

Task 1 Production and management of the G1 generation

This task was performed in 1996 and produced 3 experimental populations from crosses of 5 males and 5 females originating from 3 natural populations.

The initial project was to generate a 20 x 20 cross. This cross was performed in 1996 but the oysters had to be discarded because of a viral disease. Two similar crosses (20 males x 20 females) were attempted in 1997 with no success. Technical modifications have been made in 1997 in order to improve sanitary and technical aspects of the La Tremblade hatchery.

Task 2 Production and management of the G2 generation

This task will be performed in 1998.

Task 3 Recording of the growth performances

A first group out of the oysters from the 3 5X5 crosses performed in 1996 were individually labelled and their growth was recorded from 3 to 18 months after fertilisation. One of the objectives was to characterise the growth of oysters in order to study their physiological performance. A large range of growth performances were observed at an early stage and 100 oysters were chosen for physiological studies (see task 4).

The other main topic to be assessed this year was the maintenance of growth performance over years. Two experiments were carried out at Bouin, La Tremblade and Palavas in order to address this question.

The breeding and the rearing of the oysters was designed to generate a population (actually 3 sub-populations) with a high variability in growth. The range of the individual growth curves recorded was indeed very broad at the end of the first year. This variability was maintained over the second year. Overall, growth rate in *Crassotrea gigas* appears to be fairly well maintained over time, under labcratory conditions. This stability of the growth over time was observed under 2 different rearing conditions: low rearing density (food provided in excess, La Tremblade experiment) and competition for food (3 levels of competition for food, Bouin experiment). About 50 % of the variation observed in year 2 can be explained by the weight reached at the end of year 1.

The experiment in Bouin, where the 3 5X5 populations were reared under different levels of competition for food (high, medium and low), provides new results on variability and plasticity for growth in *Crassostrea gigas*. Oysters reared under low and medium levels of competition for food showed similar growth performances. This result was not expected (as the initial objective was to have an intermediate competition level for food in tank "2") but the similarity allows us to see that the growth performances observed are not limited by competition for food, at least for the low and medium levels of competition. We can therefore assume that the observed performances represent the maximum growth performances at the temperature of 16°C. Here we have a good estimate of the phenotypic value of growth performance for each labelled oyster. When large and small oysters were compared, in tanks "0" (low competition) and "4" (high competition), their growth was similarly affected by competition for food (reduction of growth of $\approx$ 30 %). This means that apparently there is an absence of interaction between competition level (i.e. tank) and the relationship between initial total weight and slope of growth. This might be due to the fact that, even in the high competition tank, growing conditions were still fairly intensive, and therefore not representative of field growing conditions in over-exploited sites like the Marennes-Oléron estuary in France.

Growth recordings on the oysters reared in Palavas (Thau lagoon) are still in progress,

the animals will be weighed early in 1998.

Growth rate is significantly different between 5X5 crosses however. Offspring from the Arcachon cross appear to have a lower growth rate compared with offspring from the 2 other crosses. This difference is significant in all rearing conditions (La Tremblade and Bouin). This would tend to support the idea of a genetic basis to the difference in growth between populations. However, the question remains as to what extent the observed variability can be explained by among-individual competition rather than a strong genetic basis. To answer this question, we plan to set-up a selection experiment for fast-growing and medium-growing oysters and to test these selected oysters in the same experimental scheme in 1999.

Task 4 Physiological analysis

According to the last annual report two main experiments were performed this year :

- temporal stability of physiological traits,
- characterisation of physiological performance (physiological functions, protein turn-over, proteolitic and enzyme activity) of animals from the G1 generation.

Temporal stability of physiological traits

Last year it was concluded that individual scope for growth, measured classically (discrete samples with replicates), was not a good predictor of individual growth. Indeed, the time oysters spent active, consuming oxygen and/or food, was not taken into account in such determination, even if calculation was based on the biodeposit method.

Continuous monitoring systems were developed to measure the acquisition of energy (filtration) and its loss, measured by the metabolism (oxygen consumption). This was performed in order to improve the precision of the estimation of physiological parameters (mainly the scope for growth). Such a system was developed in 1989 for respiration and more recently (September 1997) for filtration.

Experiments were performed in April, September, October and December. At present, the December experiment is not yet treated. As the continuous filtration monitoring was not working in April, only continuous oxygen consumption was recorded at this time. The observed mean value of RTA (Respiratory Time Activity: percentage of time the animal spent consuming oxygen relative to the experimental time) was in complete agreement with previous experiments. This result was included in a general paper about RTA.

Continuous filtration monitoring allowed the precise **measurement** of :

- the food availability,
- the FTA (Feeding Time Activity) defined as the time spent actually filtering

particles,

- the quantity of food filtered by the animal over a day period,
- while the classical method allowed the **estimation** of :
- the food availability, which is considered as constant between two samples,
- the filtering activity, which is estimated by observation at regular times of whether or not the valves are open,
- the quantity of food filtered, which is estimated from discrete samples and extrapolated to a day period.

Preliminary results showed a large individual variability and could be in contradiction with earlier results and from one experiment to another. Indeed, it was observed that the relationship between physiological parameters and total weight seemed to be negative in September and positive in October. Most of the oysters which presented the highest scope for growth values in September showed null or negative values in October.

At present, it could be concluded that there is no temporal stability, but as it was the first time that such measurements (rather than estimations) were made, it is difficult to explain the observed differences. The complete treatment of the five planned experiments should give more information about time activities and physiological behaviour of oysters. This will allow us to test the temporal stability of physiological traits definitively.

Characterisation of physiological performances

As planned in 1996, the same G1 animals, acclimated to temperature and low food availability, were analysed for :

- non-destructive measurements of the physiological functions,
- protein turn-over estimation,
- proteolitic activity determination,
- digestive enzyme activity measurements.

Bz peptide substrate was tested for proteolitic activity in comparison with haemoglobin substrate. Similar results were obtained with both substrates. Future analyses on GENEPHYS oysters will be done using haemoglobin substrate, as it offers greater sensitivity and ease of use.

Results showed that, except for dry weight, there is no significant differences between populations for feeding processes and for digestive enzymes.

Individual behaviour was more interesting. During this experiment a linear relationship was observed between oxygen consumption and estimated dry weight, established on

animals of the same age that had been reared in the same environment from their birth up to the experiment. Such a result is in contradiction with previously published studies. Indeed, previous authors observed an exponential relationship between oxygen consumption and observed dry weight, though this was established on oysters of different age classes.

Differences observed for digestive enzymes could be explained by the existence of two sets of animals. Oysters from the first set showed lower enzyme activities, characterised by a larger affinity to ingest food than the oysters of the second set. Such differences could be linked to growth rate.

Similarly, two groups of animals could be defined according to their relationships between scope for growth (SFG) and total weight. Inside the first group, the large SFG values were observed in larger oysters, while low SFG were measured in smaller animals. Inside the second group in contrast, high SFG values, even null or negative, were observed in small oysters, while high negative SFG were observed in large oysters.

More treatments have to be done in order to know

- the protein turn-over and proteolitic activities of fast and slow growing oysters,
- if the animals inside sets (digestive enzymes, sub-task 4.4) are the same as inside groups (SFG, sub-task 4.2).

Task 5 Genetic analyses

Genetic markers

The use of 4 highly polymorphic microsatellite loci has been standardised, allowing a safe and technically easy means of identifying parents of the progeny in mixed or mass crosses. This already accomplishes one of the main objectives of the microsatellite analysis in the project. Around 90% of parentage can be determined using only two loci. This demonstrates the efficiency of these markers for this application. Parentage identification is important for an additional reason in bivalves, it allows mixed families to be reared in a common environment in order to estimate genetic parameters. Furthermore, studies can be biased by "contamination" with "immigrants", either from the wild or from other crosses, during the very early stages of development. These contaminations happen relatively easily, and this makes microsatellites an indispensable tool for estimating their occurrence and extent.

In our crosses, the contribution of male and female parents (G0) to the next generation (G1) is highly unbalanced, as revealed by the microsatellite analysis. Some animals do not contribute to the next generation at all, whilst others contribute the majority of the progeny. This led to a considerable reduction of variation in the G1 generation and happened despite the initial effort to balance parental contributions. The experiment had originally been designed to secure equal reproductive contribution of all parents.

Although relatively large amount of genetic variation is still preserved, the above result should be taken into account in future experimental designs. From a population genetics point of view, the small original number of males and females, coupled with their unequal contribution to the next generation, is detected by a significant excess of heterozygotes in the progeny.

An uncommon observation was made in the case of a female parent, which apparently is an homozygote for one locus, yet it does not transmit any allele to several of her progeny (or if it transmits it cannot be detected). The existence of a certain proportion of individuals in the G1 generation, in which the mother's allele is not detectable in the progeny, could be confounded with the segregation of a null allele in the population. This non detection, reflecting potentially a non transmission, could be problematic for the use of this locus for parentage identification, and could also give biased results for the estimation of population genetics parameters. On the other hand we cannot exclude that this locus could be a marker of phenomena of fundamental scientific value, for a species characterised by strong segregation distortion in many loci, and loss of chromosomes.

The inheritance between the parents and the offspring of the 3 "5x5" crosses were also examined using allozyme markers. Strong deviations from Mendelian expectations were observed at various loci in the 3 populations. These deviations can be directly related to the unbalanced contributions of the parental G0 oysters in each cross. No correlation was detected between heterozygosity at allozyme loci and growth (using total weight) in the three populations. The lack of correlation between heterozygosity and growth is not an unusual observation in a restricted mating. In our crosses, populations were produced from only 10 parents so it is not surprising to find no correlation. In contrast with some studies of wild populations and another hatchery-based study involving restricted mating, no correlation was observed here between variance in size and degree of heterozygosity.

More data remain to be analysed in 1998 and the relationship between heterozygosity and growth will be examined in more detail. The integrated analysis of allozyme and microsatellite data has not yet been done, as microsatellite markers were only scored at the end of 1997.

Aneuploidy

A MgCl2 treatment, which allows the opening of the valves by relaxation of the muscles, was tested on individually labelled oysters in order to score aneuploidy on biopsies of gills (two experiments: La Tremblade 3-4 December 1996 and 13-18 March 1997). The mitosis were absent or scarce in slide preparations made from animals from the three 5 x 5 populations (Port des Barques, Bonne Anse, Arcachon). Thus aneuploidy could not be evaluated. This lack of mitosis was probably due to the non-growing period of winter in the first experiment and could be due to various problems: acclimation of animals was not successful, poor condition of the animals etc... As no non-destructive method could be developed to select broodstocks with a known aneuploidy, the study of a possible transmission of aneuploidy in the next offspring will

be done by a destructive method, i.e. immediately after spawning. This experiment is planned for Spring 1998.

The comparison between the percentage of aneuploidy in ten small animals from each population (Port des Barques 25%, Bonne Anse 25% and Arcachon 30%) and in ten large animals (Port des Barques 18%, Bonne Anse 19% , Arcachon 22%) confirms the correlation between somatic aneuploidy and growth rate. Looking at all animals studied, the total percentages of aneuploidy in the populations (which were not significantly different at p=0.05 : Port des Barques 23%, Bonne Anse 22%, Arcachon 25%) are slightly lower than those observed last year in the pseudo-cohorts (27%, 34%, 33%, 28% respectively), but still remains relatively high compared with previous results on other populations of *Crassostrea gigas* and on a control population produced at the IFREMER Argenton hatchery for which the total percentage of aneuploidy (2n= 19, 18 and 17) was 3%.

Task 6 Development of new microsatellite markers

This task was achieved in 1997. 5 new microsatellite markers were developed. One of them has been used in the analyses performed in 1997.

Task 7 Search for chromosomes markers

The search for chromosome markers is based on two different kinds of techniques : chromosome banding (G and R-bands) and FISH .

"G-bands" were performed using ASG technique in *C. virginica*, *O. edulis* and *C. gigas*. These bands allowed us to identify the different pairs of a karyotype which showed a specific pattern. "R-bands" were also performed. These bands permit a complementary diagnostic for identification of chromosomes. These results are the first on G and R-bands obtained in oysters.

A cosmid genomic DNA library was produced in 1996, containing 100,000 clones with an average insert length of 22 kbp. During this second reporting period, a larger cosmid based genomic DNA library containing 240,000 clones has been constructed with inserts ranging in length from 6 kbp to 50 kbp. Based on comparative analysis of the genome contents of other species, this genomic DNA library is estimated to contain between two and three C. gigas genome equivalents. This cloning of *G. gigas* genomic DNA is now complete. The main research emphasis of the second reporting period was the development of methods to prepare suitable chromosome spreads for C. gigas. The method has been optimised and the *C. gigas* chromosome spreads obtained appear suitable for fluorescent *in situ* hybridisation analysis using DNA probes. Method development will progress to aneuploid analysis in the forthcoming reporting period. Currently, we do not yet have FISH chromosome specific molecular markers defined for use in aneuploid analysis. However, we expect to define these cosmid markers and begin the aneuploid analysis during the upcoming third reporting period.

Task 8 Data analysis and results

This second reporting period has been mostly dedicated to the collection of data by each partner on the 3 "5 x 5" crosses. The initial multi-disciplinary approach of the GENEPHYS project - involving growth, physiology and genetics on the same biological material – is in progress, as sampling and data analysis will continue in the next reporting periods.

Task 9 General management of the project

The general management of the project is ensured by the genetic and physiology co-ordinators. The annual meeting held in L'Houmeau in November 1997 allowed each partner to be informed of all the different tasks of the project and to discuss future actions.

3. Discussion – Conclusion

This second year has yielded a large amount of data from the different partners. Growth, physiology and genetics have been investigated on common material with the same individual oysters being shared among the partners. The actions planned in 1996 for 1997 were achieved, even if all the results are not yet fully treated. Nevertheless the progress of the project shows many new and interesting results, bringing new insight on growth in oysters.

Growth recording on oysters in the experiments run in La Tremblade, Bouin (and data expected from Palavas) demonstrate that growth performance is well maintained over time from the first to the second year and that variability is very large in our biological material. The estimation of individual growth performances of 1500 labelled oysters allows us to set up the basis of a selection experiment for growth.

Genetic analyses of our 3 experimental populations gives, for the first time, results for both allozymes and microsatellite markers on the same biological material. The joint analysis has not been done but will be performed in 1998.

For the physiological analyses, it could be concluded at present that there is no temporal stability of physiological traits. More data are needed however to explain the differences observed over time. The results of the June experiment, performed on G1 oysters acclimated to temperature and low food availability, showed two different groups of behaviour both for digestive enzymes and scope for growth. A surprising linear relationship between dry weight and oxygen consumption was noticed in the June experiment.

Over all, we think that the project made significant progress during this second reporting period and that the future actions should be performed according to the initial plans.

4. Future actions

Genetical and physiological analysis on the G1 generation which will be completed in 1998 :

- growth performances recorded in Palavas,
- allozymes and microsatellites on adults,
- microsatellite analysis on larval populations,
- temporal stability of physiological traits,
- analysis of protein turn-over and proteolitic activities.

The global treatment of all the data collected by the different partners in 1997 and 1998 will be done and should provide new results of high scientific value on the genetics and physiology of growth in *C. gigas*. The comparison and the combined analysis of the results from each partner will be of great interest as different methods have been applied on the same biological material.

1998 will be mainly dedicated to the production of the G2 generations, based on physiological performances, aneuploidy level and growth of the selected parents. To estimate genetic parameters such as heritability for growth, a new G1 generation, consisting this time of full-sib families, will be also initiated. As major changes have been made in the hatchery in La Tremblade in order to improve prophylactic rearing conditions, we hope to complete this task successfully in 1998.

FAIR PL. 95.421

Methodology and research tasks

Consolidated Progress Report **for the period from** 1st January to 31st December 1997

Task 1 : Production and management of the G1 generation

Participant : 1 (IFREMER La Tremblade, Bouin, Palavas)

Duration : 36 months

Current status : 12 months to completion

Total estimated Man-months : 12 man-months

N° of man-months already devoted to the task : 16 man-months

Objectives :

Sampling of G0 parents for the production of a G1 generation with a large genetic base.

Methodology:

The zootechnical conditions during the breeding period will mainly aim to:

- maintain the whole genetic variability in the population, without any intentional selection,
- limit inter-individual competition at all stages, by working at low densities, in order to allow the expression of the full genetic variability.

Deliverable :

Production of the G1 generation.

Links:

The initial task is to establish the biological material to be studied in tasks 3, 4 and 5.

Progress in the second reporting period :

The trials to produce a second generation G1 were unsuccessful for pathology reasons. The generation G1 produced in 1996 and structured in 3 populations was managed as scheduled. In addition an experiment was run in intensive condition which had the advantage of ensuring the production of parents G1 for the next generation.

Sub-task 1.1 : Crosses between G0 parents

Participants : 1

Duration : 15 months

Current status : Completed (theoretically)

Total estimated Man-months : 9.5 man-months

N° of man-months devoted already to the task : 9 man-months

Objectives :

Establishing the G1 pseudo-cohorts.

Methodology :

In order to get a G1 generation with a large genetic base, the fundamental requirement for any evaluation of the selection potential in the species, the parents (G0) will be sampled from 4 different sites:

- the Seudre estuary ,
- the Arcachon basin,
- the Charente estuary,
- the Bonne-Anse bay (Gironde).

These sites are the main sources for spat collection for France as well as for many other European countries producing oysters commercially.

The G0 will be composed of 40 parental oysters, on the basis of 10 individuals per site (5 males, 5 females). Gametes will be obtained by stripping, and a tissue sample of each parent will be kept for further genetic analysis. In order to allow maximum mixing and the representation of each parental combination, a factorial cross (20 males x 20 females with every possible combination) will be done. The embryos born of these 400 crosses will be pooled one hour after fertilisation, and will form the G1 population.

In contrast to the usual hatchery techniques, where low-growing individuals are systematically eliminated, no selective sieving will be done so as to retain the whole population. In the first days of the breeding, large differences in growth are commonly observed among individuals.

These differences are poorly explained, and a genetical origin should be considered. The above practice will require working at low densities (1 larva/ml rather than the usual 10 larvae/ml), in order not to limit growth by inter-individual competition. Maintenance of the whole population will lead to a longer fixation period than usually observed. In order to obtain some individuals with the same date of settlement (pseudo-cohort), submerged collectors will be changed every 24 hours. The total number of pseudo-cohorts will depend on the length of the fixation period for the whole G1 population.

All the collectors will be transferred to breeding trays in Bouin IFREMER station with non-restricting trophic conditions (controlled feeding with *Skeletonema costatum*). In order to allow an optimal development of juveniles, the density on the collectors will be regulated by randomised suppression of supernumerary individuals.

Obtaining the G1 pseudo-cohorts is the initial phase which governs the following steps of the program, it will possibly require the performance of some preliminary tests so as to set up a sufficient quantity of G1 individuals of good quality.

Progress during the second reporting period

The E.C. requested the production of a second generation G1 because of high mortalities registered in the first periodic progress report. Two trials for producing a second G1 generation from two other populations (Vieux-Boucaux and Marennes) collapsed in 1997 because of hatchery mortalities due to the herpes virus. The first one was discarded between Day 13 and Day 17. The second one allowed the settlement of two sieving groups (at D18 and D23), but these were discarded after 2 months for the same reasons.

However, the surviving animals from the first G1 generation produced in 1996 are numerous enough to supply the scheduled experiments. During autumn 97, the larvae room of the hatchery was completely redesigned for better prophylactic rearing conditions. In 1998, a new G1 generation consisting of full-sib and half-sib families is scheduled in order to:

- to get enough individuals to enable future selection ;

- to supply subtask 5.3 (microsatellites on larvae) which was delayed because of bad conservation of DNA samples ;

- to verify the results on sieving groups (pseudo-cohorts) obtained in 1996.

Sub-task 1.2 :	Breeding of G1
pseudo-cohorts *Participants :*	1 (IFREMER Palavas)
Duration :	27 months
Current status :	12 months to completion
Total estimated Man-months :	2.5 man-months
N° of man-months already devoted to the task :	7 man-months

Objectives:

Breeding of the G1 pseudo-cohorts.

Materials and methods:

Removal of the oysters from collectors will be performed when they reach a mean size of 5 cm. They will then be transferred to the Etang de Thau (Méditerranée) and fixed on ropes according to local practices. The choice of this site guarantees good trophic conditions, which will give the fast growth needed to complete this project within 5 years. This breeding will be conducted for a minimum of two years.

Progress during the second reporting period

As explained in the first periodic report, this task was continued with the intra-population 5 x 5 crosses.

One year old G1 oysters were split between 2 locations in spring 97. Around 6500 individuals were transferred as scheduled to the Etang de Thau, while

1400 individuals were maintained in the intensive rearing system of IFREMER-Bouin. This was done for 3 main reasons :

- two locations were safer than only one

- good trophic conditions could be controlled and adjusted in Bouin

- selection of G1 parents for growth performances would be more efficient in a controlled environment (subtask 2.1).

The initial length of the study was planned to be 2 years but because of high growth rates, the growth performances will be recorded for only one year. The Bouin experiment was not initially planned as part of the project but at the first-year meeting it was decided that such an experiment would be of great interest for the project (this experiment has been time consuming, which explains the large number of man-months).

Task 2 : Establishment and management of the G2 generation

Participant : 1 (IFREMER La Tremblade, Bouin)

Duration : 42 months

Current status : 36 months to completion

Total estimated Man-months : 15 man-months

N° of man-months already devoted to the task : 1 man-month

Objectives :

Obtaining the G2 family.

Methodology :

Physiological and genetical analysis (Tasks 4 et 5), will allow the separation of individuals with low and high metabolic yields (if the stability of these features is proved), and of those with different genetic characteristics. Some individuals with contrasting metabolic yield, both diploids and aneuploids, will be used as parents for the G2 generation. The number of parents used will depend on the correlations observed between the different characters.

Deliverable :

These crosses will produce full-sib and half-sib famillies which will allow us to determine the heritability of the characters studied, i.e. the genetic component of the variation (additivity, dominance).

Links :

Establishment of the biological material to be studied in Tasks 3, 4 et 5.

Progress in the second reporting period :

The selection of the G1 parents (Subtask 2.1) is made possible by several operations run in 1997. The larval room of the hatchery of IFREMER - La Tremblade has been re-designed and rebuilt to ensure a higher prophylaxis for the crosses which will be done in the first semester of 1998.

Sub-task 2.1 :	Selection of G1 Parents and crosses
Participants :	1 (IFREMER La Tremblade, L'Houmeau) et 3
Duration :	15 months
Current status :	9 months to completion
Total estimated Man-months :	11 man-months
N° of man-months already devoted to the task :	1 man-month

Objectives :

To select and cross individuals with contrasting metabolic yield, and different genetic features.

Materials and methods :

A series of biparental matings will be performed between G1 individuals showing the same yield characteristics (high yield x high yield; low yield x low yield) or contrasting yield characteristics (high yield x low yield). Transmission of aneuploidy will also be studied. For each type of mating 5 males and 20 females (4 per male) will be tested (60 crosses). Gametes will be obtained by stripping and a sample of each parent will be preserved for genetical analyses (Sub-tasks 5.1 et 5.2).

Progress in the second reporting period :

Several experiments run in 97 led to the selection of G1 Parents for the production of the G2 generation in 1998 :

- G1 individuals which were studied from a physiological point of view will be crossed with one control to produce a half-sib family.

- as the tests to determine if an individual is aneuploid remain destructive, it is impossible to pre-select aneuploid G1 Parents. One population (from the IFREMER Hatchery at Argenton) has however been characterised as presenting a very low level of aneuploidy. It will be used in crosses with oysters from the genephys G1 which presents a high level of aneuploidy.

- the experiment run in Bouin to record growth-performances (cf task 3) supplys the program with labelled individuals whose growth has been characterised in low and high trophic conditions. This allows the following types to be used as parents :

  . individuals of high growth rate in low and high trophic conditions

  . individuals of medium growth rate in low and high trophic conditions

  . non-selected animals

Nota bene :In the initial program, it was mentioned that if difficulties in selecting G1 parents for physiological parameters were encountered (due to unstable values ; subtask 4.1) selection could be made on growth characteristics. In the 1997 annual meeting, all partners decided to start

studying selection for both aspects. This decision increases the work for all partners.

Sub-task 2.2 : Breeding of G2 families

Participants : 1 (IFREMER La Tremblade, Bouin)

Duration : 30 months

Current status : 36 months to completion

Total estimated Man-months : 4 man-months

N° of man-months already devoted to the task : 0 man-months

Objectives :

Establishment of the G2 families used for genetical and physiological studies.

Materials and methods :

As for the G1 generation, the G2 families will be bred in non-selective conditions (no sieving and therefore low densities) so as not to skew the assessment of genetic parameters. There are no reasons for performing the identification of pseudo-cohorts at this stage of the project, the objective is to conserve the whole genetic variability of each family. After settlement and pre-growth of the post-larvae in the hatchery (IFREMER La Tremblade), breeding will be carried out in controlled trophic and thermal conditions in the IFREMER station in Bouin.

Progress in the second reporting period :

This Subtask will start as scheduled in 1998

Task 3 : Recording of the growth performances

Participant : 1 (IFREMER La Tremblade, Bouin, Palavas)

Duration : 57 months

Current status : 31 months to completion

Total estimated Man-months : 10 man-months

N° of man-months already devoted to the task : 8 man-months

Objectives :

Recording of the growth performances of generations G1 and G2.

Methodology :

Regular measurements of the length of individuals will be made on the G1 population from the larval stage. For G2 families, the growth measurements will start after settlement. As soon as individual oysters can be identified in the G1 generation (i.e. a few weeks after settlement), an individual size recording will be made for each pseudo-cohort. A weight measurement will

be taken when they are removed from the collectors and at the time of the transfer to the Etang de Thau (Mediterranean Sea). After the oysters are stuck on the rope, only the measurement of size will be possible during the pre-growth period. Regular destructive sampling of a few individuals will allow us to determine the allometry of size/weight for each pseudo-cohort, which could be specific.
For the G2 families, the recording of growth performance (size, weight) of each family will be done in controlled trophic and thermal conditions in the Bouin IFREMER Station. An experimental protocol with 3 complete sets of repeats will be used to provide an homogeneous food supply.

Deliverable :

Growth performance of the G1 and G2 generations.

Links :

Correlations between growth performance and physiological performance will be examined (Task 4) and with genetical analysis (Task 5). The performances recorded on individuals will be used to select parents for the next generation (Sub-task 2.1).

Progress in the second reporting period :

One year old G1 oysters were split between 2 locations in spring 97 :

- A total of 6460 individuals from the 3 populations were transferred as scheduled to the Etang de Thau (Mediterranean sea) in April 1997. The experiment was designed to allow growth comparisons among different homogeneous weight classes within and between populations. Despite a dystrophic event during summer 97, growth was good with most oysters reaching a commercial size.

- 1400 other individuals were labelled and their growth was individually recorded in the intensive rearing system of IFREMER-Bouin every 2 weeks over 3 months in 3 trophic conditions (low, medium and high levels of competition). This extra experiment represents a way in which to estimate the effects of several factors on the growth performances in the second year (first year performances, change of environment, level of competition for food) and also a way to select parents G1 (sub-task 2.1).

Task 4 :	Physiological analyses
Participant :	1 et 2
Duration :	57 months
Current status :	33 months to completion
Total estimated Man-months :	45 man-months
N° of man-months already devoted to the task :	34.3 man-months

Objectives :

Recording physiological performances of generations G1 and G2.

Methodology :

The different physiological functions will be measured on adult individuals, at least one year old, for generations G1 and G2. Preliminary experiments, to check the temporal stability of physiological performance, or to perfect non-destructive measurement techniques for physiological parameters, will be performed on oysters collected in the natural environment.

Deliverable :

- Characterisation of physiological performance of the biological material studied.
- Temporal stability of physiological performance.
- Knowledge of the individual variability in physiological performance.
- Perfection of new analysis techniques (e.g. stress protein assay).

Links :

Physiological performance will be correlated with growth performance (Task 3) and genetical analyses (Task 5). Onlyindividuals which have been killed will be analysed for their allozymic and microsatellite polymorphism.

Progress in the second reporting period :

According to the last annual report two studies were performed in 1997.

1) Four experiments from sub-task 1 (temporal stability of physiological traits) were performed, and two other experiments will be done in 1998. At present, three experiments are treated, and one untreated.

2) The experiment using non-destructive measurement of the physiological functions (sub-task 2) was done in June followed by the measurement of protein turn-over (sub-task 4.3). At the end of this last experiment, animals were dissected for the analyses of proteolitic (sub-task 4.3) and enzyme (sub-task 4.4) activities. Others animal sub-samples were sent (microsatellites and allozymes) to the other partners for genetical analyses.

Sub-task 4.1 :	Analysis of the temporal stability of the physiological traits
Participants :	1 (IFREMER L'Houmeau)
Duration :	36 months
Current status :	12 months to completion
Total estimated Man-months :	6 man-months
N° of man-months already devoted to the task :	11.6 man-months

Objectives :

To study time-stability of physiological performance

Materials and methods:

In order to ensure the temporal stability of physiological performance, experiments described in *Sub-task 4.2* will be repeated during the winter, spring and autumn on the same individuals. This will only concern physiological parameters which don't require the studied animals to be killed. Until G1 oysters are big enough to be studied, preliminary experiments will be performed on oysters collected in the natural environment.

Progress in the second reporting period :

It was decided to develop continuous monitoring for clearance rate and oxygen consumption in order to improve the precision of all physiological estimations, taking into account the level of activity of the oysters. Animals would have been analysed three times in 1997, June, September and December. In April-June, the automatic sampler for measuring continuous filtration rate was not yet working, only continuous oxygen consumption was recorded. In order to improve precision in this study, 5 experiments were planned: September, October and December 1997, February and April 1998. At present, the three 1997 experiments were achieved.

Sub-task 4.2 :	Non destructive measurments of physiological traits
Participants :	1 (IFREMER L'Houmeau)
Duration :	42 months
Current status :	33 months to completion
Total estimated Man-months :	8 man-months
N° of man-months already devoted to the task :	6 man-months

Objectives :

Measurement of some of the physiological parameters without killing animals, in order to follow these oysters individually over time and to be able to select them according to their performance.

Materials and methods:

The different physiological functions will be measured in adult G1s and G2s. Some measurements (definitely metabolism and food consumption), will be performed for a 12 hour period in order to determine their level and the daily duration of their activity.
For generation G1, 3 pseudo-cohorts will be analysed at the rate of 50 individuals per set of s.
For G2, the large number of families will need preliminary treatment of all types of data obtained in the G1 in order to select the more pertinent physiological traits and to measure them at the family level. It is under consideration however, to focus analysis effort on a few representative families, in order to obtain the full genetical, physiological and growth data. Between 20 and 30 individuals will be analysed per family retained.
Non-destructive physiological functions measured are :

-oxygen consumption,
-ammonium excretion,
-quantity of food consumed,
-ingestion rate,
-absorption rate,
-scope for growth.

These measurements will be made in an experimental tank.and will need to be done before the allometry rates are calculated on the same populations.

Progress in the second reporting period :

According to the last report (1996), animals from three intra-populations were studied for physiology in a non-destructive manner. At the end of this experiment (June 1997), Partner 2 did the protein turn-over experiment. The animals were then dissected and sub-samples were prepared for physiological (proteolitic(sub-task 4.3) and enzyme (sub-task 4.4) analyses) and genetical (microsatellite and allozymes) analyses. Data treatments remain to be completed.

Sub-task 4.3 :	Proteolytic activity and proteic turn-over measurement
Participants :	2
Duration :	42 months
Current status :	33 months to completion
Total estimated Man-months :	27 man-months
N° of man-months already devoted to the task :	10.7 man-months

Objectives :

- Proteolitic activity measurement, which is used to identify lysosomal and non-lysosomal degradation processes in different tissues.
- Proteic turn-over measurement (total protein synthesis proportion which affects the renewal and the replacement rather than the net gain).
- Measurement of the proteic turn-over regulation. This is to evaluate the amount of stress protein which takes part in the regulation of the spatial conformation of proteins, their translocation and their degradation.

Materials and methods:

Measurements will require killing the individuals to be analysed. From the generation G1, the 3 pseudo-cohorts will be then analysed according to various techniques using20 to 30 individuals per pseudo-cohort. Analysis dealing with allozymic and microsatellite polymorphism will be of course performed jointly by partners 4 and 5.

As in the previous Task, the high number of G2 families will require a preliminary treatment of all types of data to select the most pertinent physiological traits in order to measure them on whole families. It is however under consideration to focus analysis effort on a few representative families,

in order to obtain the full genetical, physiological and growth data. These analyses will be performed concurrently with ecophysiology experiments on the individuals used for the calculation of the allometry rates. A maximum of 20 to 30 individuals per family will be analysed.
Direct measurement of the level of maintenance metabolism will use recently developed techniques. These are based on the use of the stable nitrogen isotope N15 to determine the proteic turn-over as the proportion of the total protein synthesis affecting the renewal and replacement, rather than the net gain. This method also provides a measurement of the protein gain efficiency, defined as the percentage [(proteic gain/total protein synthesis) x 100], as well as a quantification of the relative recycling of the protein degradation products as a function of its synthsis.
Development and application of methods measuring proteolytic activity will be used in order to differenciate the lyse processes due to lysosomes. These techniques are already used on the blue mussel (*Mytilus edulis*) and the results suggest a strong influence of genetic and seasonal factors on proteolytic activity. After adaptation of the technique to *C. gigas*, proteinase cathepsine and leucine aminopeptidase will be measured on fragments of mantle or digestive gland. Specific activity will be calculated as enzyme unit per milligramme of total protein. For leucine aminopeptidase, enzymatic unit will be defined as the quantity producing an increase in absorbance of 1.0 per minute. For proteinase cathepsin B and D a unit will be the equivalent, respectively, to 1 nmole of product per minute and 1 µmole of product per minute.
Stress protein measurement has also been developed in *Mytilus edulis* by immunological detection of 4 protein isoforms. After polyacrylamide gel electrophoresis and Western-blotting analysis, specific monoclonal antibodies of the "ATP-binding" region of the stress protein P-70, will be used to select proteins of apparent molecular weight 70, 72 and 78 Kda. This technique is not well defined yet, so it is not possible to evaluate how many analyses will be performed.

Progress in the second reporting period :

To rule out the possibility that our findings for Cathepsin D were due to artefacts associated with our choice of substrate, we have developed an assay that uses a synthetic peptide substrate, and compared results with those obtained for haemoglobin. We have also undertaken our first collaborative experiments whilst working with IFREMER scientists in France to compare protein of the three G1 populations

Unfortunately, samples of adductor muscle, digestive gland and gill destined for analysis of proteolytic enzymes and stress proteins were lost in transit from IFREMER to PML due to a faulty cool-box

Therefore, in July 1997, a further sixty oysters were dissected from G1 that had been monitored for growth at Bouin.

Sub-task 4.4 :	Digestive enzyme activity measurement
Participants :	1 (IFREMER Brest)
Duration :	42 months
Current status :	33 months to completion
Total estimated Man-months :	6 man-months
N° of man-months already devoted to the task :	6 man-months

Objectives :

- Search for correlation between specific activity of digestive enzymes, ingestion, assimilation performance and growth.
- Assess whether some individuals, show different levels of specific activity for digestive enzymes and correlated differences in assimilation yields and growth, when faced with the same nutritional signal.

Materials and methods:

Digestive enzyme analyses will be performed on surpernatant from spun down crushes of the digestive gland (juveniles and adults), or of the whole animals (larvae and post-larvae). Activities of two carbohydrases (amylase et laminarinase) will be measured by techniques perfected on the Alliance auto-analyser, which enable us to treat a large number of samples. Amylase will be assayed according to a protocol adapted for molluscs, by reaction of the extract prepared in 0.02M $CaCl_2$, 0.2M NaCl on 1mg/ml starch at pH 6.2 and 45°C. Starch disappearance is measured using iodure and IK method. Laminarinase will be assessed according to the method perfected for molluscs in the lab, by making aqueous extract act on 2 mg/ml laminarin at pH 4.8 and 35°C. Reducing sugar appearance is measured by the neocuproine technique. Total protein from the sample is extracted overnight with 0.1N sodium hydroxyde at 4° C and assessed according to the Lowry method using BSA standard in 0.1 N sodium hydroxyde.
These measurements will be carried out from the G1 generation lots (3 pseudo-cohorts with 50 individuals per lot) after the non-destructive experiments (ingestion and assimilation performances) have been performed. For the generation G2, a first estimate of features of the products will be performed on 15 day old larvae (3 to 5 replicates). Then, according to the retained protocol, analyses will be performed on each lot for which we have data for ingestion and assimilation performances, and on animals killed for their genetical characterisation (20 to 50 individuals per lot, each grown under strictly identical food conditions in the IFREMER Station in Bouin).

Progress in the second reporting period :

Analyses of G0 generations demonstrated that seasonal differences were observed in digestive equipment according to our knowledge on relationships between digestive enzyme activities and food ingestion. Differences observed between origins were attributed to heterogeneity in age between origins (Seudre and Bonne Anse). A large identical standard deviation observed in the four populations was attributed to a large genetic diversity

and phenotypic adaptations to diverse trophic environments and ages.

Analyses of the three G1 populations demonstrated that the means of digestive activities were not significantly different and that standard deviation was reduced in relation to controlled feeding conditions and age of these oysters. However, the 60 data demonstrated a large variability in absorption yield versus ingestion. By introduction of digestive enzyme activities, ingestion rate and two different constants of affinity in a Michaelis-Menten like model, we obtained a high correlation between predicted and observed absorption yield.

This model suggested that at least two sub-populations existed in this set of data. One demonstrated a high affinity for food, leading to a high absorption yield at low food ingestion, the other demonstrated a low affinity for food, leading to high absorption yield only when a high level of food was ingested .

The two sets of samples were separated. Animals with high affinity had a lower digestive equipment than the others and a different growth rate distribution with ingestion which can be interpreted by the model.

We suggest testing if differences in affinity are related to differences in digestive enzyme affinities. This will be performed on the same sample in 1998. We have identified different genes of amylase in different individuals. This should be studied in relation to the observed results.

Task 5 :	Genetic analyses
Participant :	1, 3, 4, 5 et 6
Duration :	57 months
Current status :	33 months to completion
Total estimated Man-months :	100 man-months
N° of man-months already devoted to the task :	60.6 man-months

Objectives :

Different hypothesis stand between "global effect" supporters (associative superdominance, aneuploidy) and "local effect" supporters (direct superdominance, null alleles, imprinting) with regard to the relationship between heterozygosity and growth. By using molecular markers (microsatellites) in parallel with enzymatic markers and karyotype analysis on different pseudo-cohorts of the population G1, we intend to provide new perspectives on this debate.
This project also goes further, since it pays particular attention to correlation between different physiological and genetic traits and not only to growth.

Methodology :

Different techniques (allozymes, microsatellites, karyology) will be used to aim to correlate physiological performances, growth performances and genotypes.

Expected results:

1-Level of global polymorphism for the G0 and G1 generations with microsatellites and allozymes.
2-Differentiation between parental populations (to be compared with existing allozyme data).
3-Real contribution of G0 parents to the G1 generation by using microsatellites.
4-Global heterozygosity of the G1 population in relation to physiology and growth, and comparison between different pseudo-cohorts.
5-Emphasis on possible deficit of heterozygotes: changes over time at the level of whole population (larvae, post-larvae, juveniles, adults), comparison between different pseudo-cohorts.
6-Comparison of the results obtained using microsatellites and allozymes, at the different levels of analysis.

Links :

Correlation will be studied between the results of genetical analyses , growth performance (Task 3) and physiological performance (Task 4).
Improvement of techniques will be carried out in Tasks 6 and 7.

Progress in the second reporting period :

Genetic analyses were made on G1 juveniles and G0 parents with microsatellites and allozymes (subtasks 5.1 and 5.2). Karyotypes were studied on larvae (in 1996) and juveniles (in 1997), but a non-destructive karyologic method has still to be developed. Microsatellites will have to be studied on larvae of the second G1 which is to be produced in 1998 (subtask 1.1) as conservation problems were encountered with the samples of 1996.

Sub-task 5.1 :	Allozymes
Participants :	4
Duration :	36 months
Current status :	12 months to completion
Total estimated Man-months :	14 man-months
N° of man-months already devoted to the task :	15 man-months

Objectives *: (see above)*

Materials and methods:

Parents and juveniles will be analysed for their allozymic polymorphism. Variability at the studied loci will be deduced from the study of the 40 G0 parents. Larval and post-larval stages are not suitable for allozymic analysis owing to their small size. Analyses will be performed systematically on G1 juveniles and adults. Analysis will be carried out on 100 juveniles from 3

different pseudo-cohorts and on 200 adults from the same pseudo-cohorts (300 and 600 individuals respectively).

Progress in the second reporting period :

Some problems encountered in the sending of samples reduced the number of analysis down to 50 juveniles per population of the generation G1. The results show that there are some deviations from Mendelian expectations in the juvenile sample and that no correlation is evident between heterozygocity and growth or heterozygote deficiency.

Sub-task 5.2 :	Microsatellites on adults and juveniles
Participants :	5
Duration :	36 months
Current status :	12 months to completion
Total estimated Man-months :	38 man-months
N° of man-months already devoted to the task :	22.6 man-months

Objectives :(see above)

Materials and methods:

Juveniles and adults will be analysed for the polymorphic microsatellite loci available.
Just like allozymes, analyses will be performed with a minimum of 3 microsatellites on the 40 G0 parents, 100 G1 post-larvae and 200 G1 adults per pseudo-cohort (this will be a total of 940 individuals). Genetic analysis of the generation G2 will depend on the results obtained on generation G1.
Up to now, 3 microsatellite loci have been identified for *Crassostrea gigas* (2 by Dr A. Magoulas in the Contrat AIR1 N°3003 92 0168 and 1 by Dr D. Hedgecock (Bodega Marine Laboratory, Davis University, California)). We will ensure the availability of new microsatellite loci by creating a new DNA bank and screening it(*Task 6, Partner 6*).

Progress in the second reporting period :

For each of the 3 remaining 5x5 factorial crosses made in 1996, 4 loci (the 3 already available and 1 new) have been studied in the 10 parents and in 50 juveniles. Parentage has been identified for 146/150 juveniles among which only 2 were identified as "contaminants". The efficiency of the 4 loci for parentage identification is now proven.

The contribution of different parents in the crosses appears to be very heterogeneous, which should be taken into account in the data processing.

Sub-task 5.3 :	Microsatellites on larvae
Participants :	6 et 1 (Partner 6 leader of this Sub-task)
Duration :	42 months
Current status :	18 months to completion
Total estimated Man-months :	20 man-months
N° of man-months already devoted to the task :	5.3 man-months

Objectives:

Extraction and PCR amplification of DNA of individual larvae, scoring of microsatellite polymorphism for the population genetics study of larvae: heterozygote deficiency, growth-heterozygocity relationships. Microsatellite markers obtained by Partner 6 will be used in this Sub-task.

Materials and methods:

Recent papers show that the PCR analysis of a sample consisting of 10 larvae can be successfully done (Banks *et al.*, 1993). The present methods to extract oyster DNA are based on gill samples of adult individuals (phenol-chloroform method).DNA extraction from a single larva require this method to be modified for a whole individual, knowing that the digestive gland tends to damage the DNA.
Analyses will be done on 3 sets of 200 G1 larvae, sampled at different times: 24 hours after crossing (J1), at the settlement stage (J20), and when post-larvae can be removed from the collectors (J45). Depending on the results, analysis will be performed on some of the G2 families, in order to study segregation distortion.

Progress in the second reporting period :

Problems were encountered in the conservation of the samples of the 1996 crosses. For this reason this sub-task is delayed to 1998. It will be done on larvae of the second G1 generation, produced in 1998 (subtask 1.1). Nevertheless, the technique has been developed and was tested on haploid and diploid embryos.

Sub-task 5.4 :	Karyotype analysis
Participants :	3 et 1 (Partner 3 leader of this Sub-task)
Duration :	57 months
Current status :	33 months to completion
Total estimated Man-months :	28 man-months
N° of man-months already devoted to the task :	17.7 man-months

Objectives :(see above)

Materials and methods:

Ovocyts from each G0 female will be sampled for karyotype analysis. Chromosomes will be visualised with Hoechst 33258 by using an epifluorescence technique, perfected by Dubé *et al.* (1985). Detection of any aneuploidy at the gamete level will be done from the parental stage. Unfortunately, because of the condensation of chromosomes, such an analysis will not be accessible with sperm.
Karyological analysis of G1 embryos (24 hours after fertilization) will be performed, on the spat, according to a method based on that of Thiriot-Quiévreux & Ayraud (1982).
First analyses in different generations will be done as soon as oysters reach a minimum size of 1 cm. Aneuploidy rate will be then assessed on samples from each (G1) pseudo-cohort or (G2) family.
Three experiments will be done :
1-The first experiment requires the killing of some animals and will consist of numbering chromosomes on gills of oysters from different G1 pseudo-cohorts. A preliminary analysis consists of correlating aneuploidy rate with the pseudo-cohort's rank, which could constitute an original result.Later, oysters of different sizes will be sampled within each pseudo-cohort in order to check if the aneuploidy rate is correlated with the size of animals, as shown in previous studies.
2-The second experiment will be done on older G1 animals, and will consist of the identification and marking of some animals of known aneuploidy rate in order to rear them to sexual maturity and to perform further matings.
3-The third experiment will consist of checking aneuploidy transmission in the G2 families.

Progress in the second reporting period :

No destructive method to select broodstocks with a known aneuploidy was developed because of the lack of mitosis in 2 experiments on 120 individuals. The estimation of aneuploidy among the three 5x5 crosses revealed lower percentages of aneuploids in the 72 juveniles studied than in the sieving groups studied in 1996, but the results were still high compared to a control population. The aneuploidy of the G1 parents used for one of the G2 generation (crosses between these populations) and the aneuploidy of their offspring will be scored in 1998.

Positive results were achieved in the use of banding techniques for the identification of oyster chromosomes.

Task 6 :	Development of new microsatellite markers
Participant :	6
Duration :	48 months
Current status :	24 months to completion

Total estimated Man-months : 20 man-months

N° of man-months already devoted to the task : 16.6 man-months

Objectives :

To obtain new microsatellite markers in order to estimate the genetic polymorphism and the heterozygosity rate in the individuals studied. A minimum of 5 loci are required, and 10 (one per chromosome) would be desirable.

Methodology :

Identification of new satellites will be done by building a new genomic bank. The genomic bank will be screened with different oligonucleotides in order to detect clones containing tandem repeat patterns, i.e."microsatellites". These will be sequenced to determine flanking sequences of repeat patterns so as to define primers which will enable us to amplify them with PCR.

Deliverables :

New microsatellite markers.

Links :

Sub-tasks 5.2 et 5.3

Progress in the second reporting period :

The development of a new marker was mentioned in the first progress report. This result was validated in 1997 by the fact that partner 5 used it on juveniles and adults (subtask 5.2).

Task 7 : Search for chromosome markers

Participant : 4

Duration : 54 months

Current status : 30 months to completion

Total estimated Man-months : 24 man-months

N° of man-months already devoted to the task : 10 man-months

Objectives :

Attempting to identify missing chromosomes in aneuploids. The underlying hypothesis is that different classes of aneuploids are not equivalent because of the *a priori* different contribution of each chromosome. Unfortunately, different chromosomes cannot be distinguished from each other by classical methods used in karyology. The *in situ* hybridisation technique (FISH) will be perfected with homologous probes, in order to obtain at least one specific probe per chromosome.

Methodology :

Fluorescent molecules will be hybridised on chromatin (chromosomes from cells fixed on slides, according to conventional technique of "chromosome

banding") in sites corresponding to a specific sequence of DNA using the *in situ* fluorescence hybridisation technique (FISH).
Probes used are single copy sequences, large size inserts in cosmids or "YACs" (yeast artificial chromosomes). DNA or RNA sequences of the probe are labelled with "reporter" molecules using "nick translation", or potentially PCR. Probes and target chromosomes are denaturated, and complementary sequences will reanneal. After rinsing and incubating in a fluorescent labelled reagent, a fluorescent signal is visible with a microscope on the chromosome where the probe has hybridised.
If this technique is successfully perfected, individuals detected as aneuploids (Sub-task 5.3) will be analysed by *in situ* hybridisation in order to identify the missing chromosomes.

Deliverables :

To obtain specific probes for each chromosome of *C. gigas*

Links :

Sub-tasks 5.4

Progress in the second reporting period :

A second cosmid-based genomic DNA library was constructed in 1997 and is larger than that of 1996. A method has been optimised to prepare suitable chromosome spreads for fluorescent *in situ* hybridisation analysis. Once the problems of background hybridisation, which are probably caused by the use of human DNA as competitor are solved, it is expected that it will be possible to define specific cosmid markers, which should allow the start of the aneuploid analysis .

Task 8 :	Data processing and results synthesis
Participant :	1, 2, 3, 4, 5 and 6.
Duration :	54 months
Current status :	36 months to completion
Total estimated Man-months :	30 man-months
N° of man-months already devoted to the task :	12.3 man-months

Objectives :

- Data analysis and results synthesis.
- Writing of intermediary reports and the final synthesis.

Methodology :

Correlations between heterozygosity, aneuploidy, physiological performance and growth performance will be demonstrated using histograms and mean comparisons. The relation between growth and different physiological traits will be characterised either by sensitivity analysis of growth models, or by multivariate analysis with PCA type or linear or non- linear multiple correlation analysis. Addad, Seneca 2.0 or SAS software will be used.

Analysis of the genetical component of the variance for all the traits measured will be performed on the second generation by variance analysis, by using quantitative genetic theory applied to hierarchical mating plans and by using SAS.
Data processing will be co-ordinated by Partner 1, IFREMER La Tremblade for the genetics and IFREMER L'Houmeau for the physiology.
Each year a global report of the progress of the work will be provided by the co-ordinator of the project, to which will be added the individual contributions of the contracting partners who participated in these tasks. Scientific publications written from these results obtained within this project will be also provided.

Links :

Results synthesis from Tasks 1, 2, 3, 4, 5, 6 et 7.

Progress in the second reporting period :

Up to now data processing has been done by each partner. Co-ordinated data processing will be made at the end of the main tasks on the G1 generation.

Task 9 :	General organisation of the project
Participant :	1, 2, 3, 4, 5 and 6.
Duration :	60 months
Current status :	36 months to completion
Total estimated Man-months :	15 man-months
N° of man-months already devoted to the task :	7.8 man-months

Objectives :

- General organisation of the project.
- Discussion and development of protocols.
- Co-ordination of tasks between the different partners.
- Presentation and results analysis of each partner.
- Preparation and writing of the intermediate and final reports as well as the valorisation as congress presentations and publications in scientific and technical reviews.
- Financial management of the project.

Methodology :

A first meeting for organisation of the project took place after the signing of the contract in order to co-ordinate the different tasks and to polish up protocols. At the end of each year, a bilingual meeting will be organised in order to focus on the progress report of the program and to prepare technical annual reports for the commission. Each year, regular meetings will be organised between different partners linked by tasks or sub-tasks to improve protocols. At the end of the project, a last meeting will be organised in order

to make a synthesis of the whole project and to prepare the final document to be submitted to the Commission.

Links :

Links with all the other tasks 1, 2, 3, 4, 5, 6, 7 et 8.

Progress in the second reporting period :

Several bi- and tripartite meetings were organised during the year to plan experiments precisely. Most of the time, preliminary work had been done through E-mail for maximum efficiency. A lot of labour was required to prepare samples and to package them for sending to all partners. These tasks of common interest were not especially difficult but were time-consuming. The annual meeting with all partners took place in La Rochelle on 13-14 November 1997.

Milestone

The following table sums up the progress status of each sub-task with regard of the scheduled project :

Operation	in preparation	started	running	achieved
Sub-task 1.1		as scheduled	extended at the EC demand	
Sub-task 1.2		as scheduled	as scheduled	
Sub-task 2.1		as scheduled	as scheduled	
Sub-task 2.2	as scheduled			
Task 3		as scheduled	as scheduled	
Sub-task 4.1		as scheduled	as scheduled	
Sub-task 4.2		as scheduled	as scheduled	
Sub-task 4.3		in advance	as scheduled	
Sub-task 4.4		in advance	as scheduled	
Sub-task 5.1		as scheduled	as scheduled	
Sub-task 5.2		as scheduled	as scheduled	
Sub-task 5.3		as scheduled	as scheduled	
Sub-task 5.4		as scheduled	as scheduled	
Task 6		as scheduled	as scheduled	
Task 7		as scheduled	as scheduled	
Task 8		as scheduled	as scheduled	
Task 9		as scheduled	as scheduled	

Deliverables

The following table sums up what techniques and biological materials have been delivered or are to be delivered

	TECHNICAL DELIVERABLES		BIOLOGICAL DELIVERABLES	
Operation	delivered	*to be delivered*	delivered	*to be delivered*
Task 1			first generation G1	*second generation G1 (1998)*
Sub-task 2.1			parents selected on growth performances	*parents selected on physiological performances (1998)*
Sub-task 2.2			(not started)	*(not started)*
Task 3			Growth of first G1	*Growth of second G1*
Sub-task 4.1				*temporal stabilty of performances (1998 for G1)*
Sub-task 4.2	Non destructive measurement protocols of physiological traits			*Non destructive measures of physiological traits (1998 for G1)*
Sub-task 4.3				*in progress*
Sub-task 4.4				*in progress*
Sub-task 5.1			allozymes on G0 and juveniles G2	*allozyme on adults G1 (1998)*
Sub-task 5.2			microsatellites on G0 and juveniles G1	*microsatellites on adults G1 (1998)*
Sub-task 5.3	microsatellites tested on embryos			*microsatellites on larvae of second G1 (1998)*
Sub-task 5.4	banding techniques		aneuploidy of G1 and control populations	*aneuploidy of the parents G1 used for generation G2 (1998)*
Task 6	5 new microsatellite markers			
Task 7	cosmid genomic DNA library			
Task 8		*Treatment of G1 data (1998)*		
Task 9	1st and 2nd intermediate progress reports	*intermediate synthesis (1998)*		

Dissemination

Posters and Oral presentation

Collet B., Boudry P., Heurtebise S., Morand B. et Gérard A. (1996) Les relations entre la date de fixation, la croissance et l'hétérozygotie en un marqueur microsatellite chez l'huître creuse *Crassostrea gigas*. XIX ème colloque de biologie et génétique des populations, Perpignan, 2-5 sept 1997.

Collet B., Boudry P., Bougrier S., Heurtebise S., Phélipot P., Ledu C., Morand B., Héral M. et Gérard A (1997). Etude des bases génétiques et de la variabilité des caractères physiologiques impliqués dans la croissance chez l'huître creuse *Crassostrea gigas*. Journées conchylicoles 1997 IFREMER Nantes, March 18-19 1997

Publications

Bougrier S., Collet B., Geairon P., Geffard O., Héral M., Deslous-Paoli J.M. (1998) Respiratory time activity of the Japanese oyster *Crassostrea gigas* (Thunberg). J. Exp. Mar. Biol. Ecol., 219(1-2): 205-216.

Collet B., Boudry P., Thebault A., Heurtebise S., Morand B. and Gérard A. Relationship netween pre- and post-settlement growth in the Pacific oyster *Crassostrea gigas* (Thunberg), **in prep**.

Hawkins, A. J. S. & Day, A. J. (1998) Metabolic interrelations underlying the physiological and evolutionary advantages of genetic diversity, **Submitted**

Abstracts

Hawkins, A. J. S. & Day, A. J. (1997) How protein metabolism can help explain the physiological and evolutionary consequences of genetic variation. American Zoologist, 37A: 9A.

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°1

IFREMER
Institut Français de Recherche pour l'Exploitation de la Mer

Laboratoire Génétique Aquaculture et Pathologie
La Tremblade
Centre de Recherche en Ecologie Marine et Aquaculture,
L'Houmeau
Laboratoire de Physiologie des Invertébrés Marins,
Brest
Laboratoire Conchylicole de Méditerranée,
Palavas

Reporting Period:
1st January-31st December 1997

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°1

Total cost to partner n°1 : 1.216.745 ECU

EC contribution : 370.000 ECU ***% of the total cost :*** 30.4

Duration : 60 months

Commencement date : 1/1/1996
Completion date : 31/12/2000

EC contact : DG XIV / C.2 (Fax : (+32-2)295.78.62

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Coordination genetic aspect :
Dr Pierre BOUDRY
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Coordination physiological aspect :
Dr Serge BOUGRIER
(IFREMER L'Houmeau)

Participant n°1 : IFREMER

Participant n°1a IFREMER – Laboratoire GAP BP 133 17 390 La Tremblade (France)	Coordinator	Dr. André GERARD Dr Pierre BOUDRY
Participant n°1b :IFREMER – CREMA Place du Séminaire, BP 7 ,L'Houmeau 17 137 Nieul-sur-mer (France)		Dr Serge BOUGRIER
Participant n°1c : IFREMER – Palavas Chemin de Maguelone 34250 Palavas-les-Flots (France)		Dr Dominique BUESTEL
Participant n°1d : IFREMER – Brest BP 70 , 29280 Plouzané (France)		Dr Jean-François SAMAIN

FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1997

Participant n°1 **IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer)**

Laboratoire Génétique, Aquaculture et Pathologie (GAP)
BP 133
17390 La Tremblade - France

Scientific team

Laboratoire Génétique Aquaculture et Pathologie
(IFREMER La Tremblade and Bouin) (Partner : P1a)
Dr. André GERARD,
Dr. Pierre BOUDRY,
Dr. Claude DELSERT
Emmanuel GOYARD
Jean-Pierre BAUD (Bouin),
Serge HEURTEBISE,
Christophe LEDU,
Max NOURRY (Bouin),
Pascal PHELIPOT.

Centre de Recherche en Ecologie Marine et Aquaculture
(Unité mixte IFREMER/CNRS L'Houmeau) (Partner : P1b)
Dr. Maurice HERAL,
Dr. Serge BOUGRIER,
Dr. Pierre GAREN,
Hervé CHARTOIS,
Bertrand COLLET (PhD),
Florence CORNETTE,
Françoise MORNET.

Laboratoire Conchylicole de Méditerranée
(IFREMER Palavas) (Partner : P1c)
Dr Dominique BUESTEL,
Catherine VERCELLI,
Jocelyne OHEIX.

Laboratoire de Physiologie des Invertébrés Marins
(IFREMER Brest) (Partner : P1d)
Dr Jean-François SAMAIN,
Jeanne MOAL,
Claudie QUERE,
Jean Yves DANIEL.

Objectives :

- Obtaining and managing the biological material
- Recording growth of the biological material
- Participation in genetical analysis
- Measurement of physiological function
- Data synthesis and exploitation.

Actions in the project :

Task 1	Obtaining and management of the G1 generation
Sub-task 1.1	Parent G0 crossbreeding, realisation of the whole sub-task by the P1a Partner.
Sub-task 1.2	Breeding of the pseudo-cohorts G1, realisation of the whole sub-task by the P1c Partner.
Task 2	Obtaining and management of the G2 generation
Sub-task 2.1	Selection of the G1 parents and crossbreeds, a)parents sorting on physiological criteria by the P1b Partner, b)parents crossbreeds by the P1a Partner.
Sub-task 2.2	G2 families breeding, realisation of the whole Sub-task by the P1a Partner.
Task 3	Recording of growth performance realisation of this Sub-task by the P1a and P1c Partner.
Task 4	Physiological analyses
Sub-task 4.1	Temporal stability analysis of physiological traits, realisation of the whole sub-task by P1b partner.
Sub-task 4.2	Non destructive measurements of the physiological functions, realisation of the whole sub-task by the P1b partner.
Sub-task 4.4	Measurement of digestive enzyme activity, realisation of the whole sub-task by the P1d partner.
Task 5	Genetical analyses
Sub-task 5.3	Larval stage microsatellites (Partner P1a) Collaboration with partner 6 (leader of the whole sub-task) for the perfectionment of the extraction method and amplification DNA from individual method.
Sub-task 5.4	Karyotype analyses (Partner P1a) a)Karyotype analyses in ovocyts, b)Study of the aneuploidy transmission (with Partner 3).
Task 8	Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis. Co-ordination by the Partner 1 of this Task.
Task 9	General organisation of the project Co-ordination of this Task by Partner 1..

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
1.1	Crossbreeding/ RP				FR
1.2	G1 breeding/ RP	Breeding G1 / RP	Breeding G1 / IS		FR
2.1		Parents assortment /RP	Crossbreeding/ RP		FR
2.2			Breeding G2 / RP	Breeding G2 / RP	Breeding G2 / FR
3	Data acquisition G1/ RP	Data acquisition G1/ RP	Data acquisition G1 et G2 / IS	Data acquisition G2/ RP	Data acquisition G2 / FR
4.1	Physiological measurement / RP	Physiological measurement G1/ RP	Physiological measurement G1 / IS		FR
4.2		Physiological measurement G1/ RP	Physiological measurement G1 / IS	Physiological measurement G2/ RP	Physiological measurement G2 / FR
4.4		Physiological measurement G1/ RP	Physiological measurement G1 / IS	Physiological measurement G2/ RP	Physiological measurement G2 / FR
5.3	Perfectionment of the technique / RP	Perfectionment and application / RP	Lab analyses / IS	Lab analyses / RP	FR
5.4	Ovocyts analyses G0 / RP		IS	Study of the aneuploidie transmission / RP	Study of the aneuploidie transmission / FR
8	Data processing and statistical analyses / RP	Data processing and statistical analyses / RP	Data processing and statistical analyses / IS	Data processing and statistical analyses / RP	Data processing and statistical analyses / FR
9	Coordination + 2 meetings with all Partners	Coordination + 1 meeting with all Partners	Coordination + 1 meeting with all Partners	Coordination + 1 meeting with all Partners	Coordination + 1 meeting with all Partners

PR = Periodical report
IS = Intermediary Synthesis
FR = Final Report

Task 3: Growth recording

Sub-task: Growth analysis: correlation between year 1 and year 2.

1. Growth recording in La Tremblade

1.1. Introduction

I. Growth is a multivariate parameter and is highly variable among individuals. This task aims to study this variability from the analyse of the population created last year. The temporal stability of the growth in *Crassotrea gigas* was investigated in order to estimate the correlation between the growth in year 1 and the growth in year 2. This task was Initially planned to be carried out on the sieving groups (pseudo cohorts, see technical annexe). Because of massive mortility due to the herpes-like virus, only the early spat growth could be analysed. An effect of the date of settlement on early spat growth rate could be pointed out (Collet et al., in preparation).

1.2. Material and methods

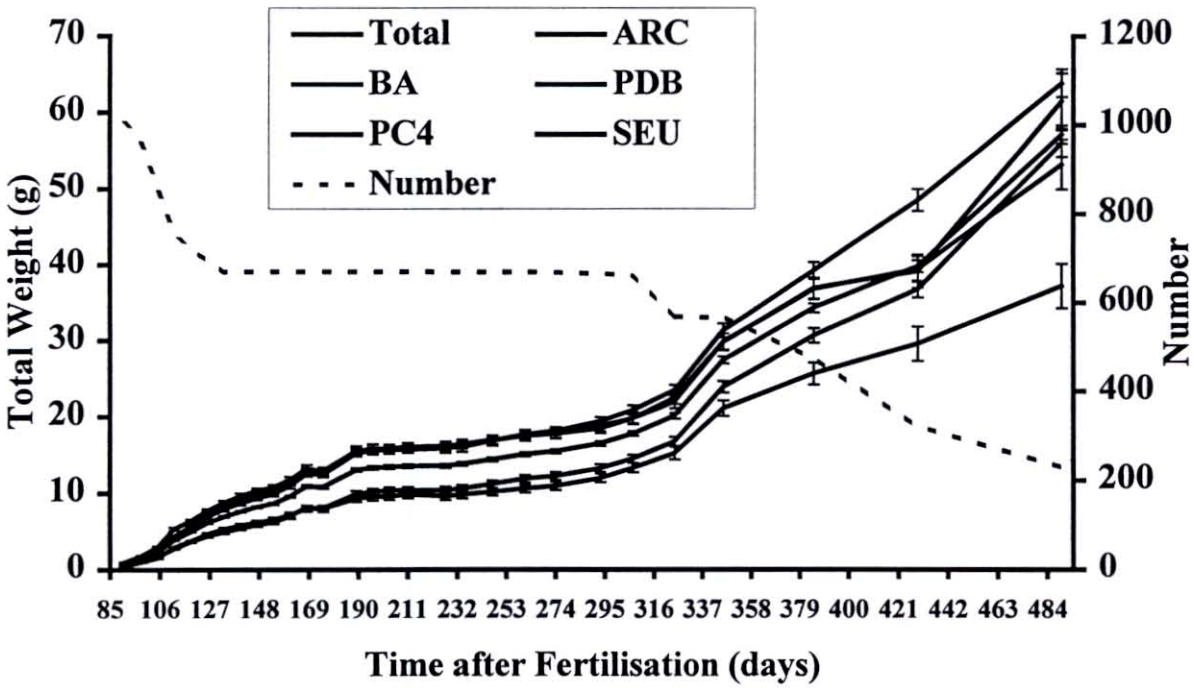
Four populations coming from the 5X5 crosses and the 4th pseudocohort coming from the 20X20 crossbred performed in April 1996 were recorded for growth under controlled condition. The density and the amount of food was maintained as constant as possible. 3 months after fertilisation, the animals were identified by using their location (column and row numbers) in a special microtank structure (see first year report). The structures were maintained in 300-liter raceways with *Skeletonema costatum* enriched sea water. 72 individuals per structure and four structures per raceway were used. Animals within a structure were randomly set out. The total weight was recorded every week, and the structures were randomly arranged again in the raceways in order to randomize the effect of the raceway on growth. A total of 1213 animals were recorded. When the animals were large enough, they were labelled and transferred in 800-liter tanks. In this way, the growth was recorded from 3 months after the cross to 18 months. All the data analyses were performed by using SAS software. PROC GLM was used for analyses of the covariance and analyses of the variance.

All the remaining oysters were used for several purposes: 75 oysters were used for the protein turn-over and stress protein experiments (Task 4.3., partner 2) following the determination of the non-destructive physiological performances (Task 4.2., partner 1). Samples for digestive enzymes (Task 4.4., partner 1), genotyping using microsatellites (Task 5.2., partner 5) and allozymes markers (Task 5.1., partner 4). 120 oysters were used for the study of the temporal stability of the physiological traits (Task 4.1., partner 1). All the samples both experimental and recently dead animals were sampled for DNA analyses.

1.3. Results and discussion

The last growth kinetics and the number of individuals is given in Figure 1 in terms of time after fertilisation.

Figure 1: Growth kinetics and number of the animals recorded in 1996 and 1997. ARC, BA, SEU, PDB represents the 5X5 populations, PC4 represents the 4th pseudo cohort of the 20X20 population. The Total is the whole population. Data are mean ± SE.

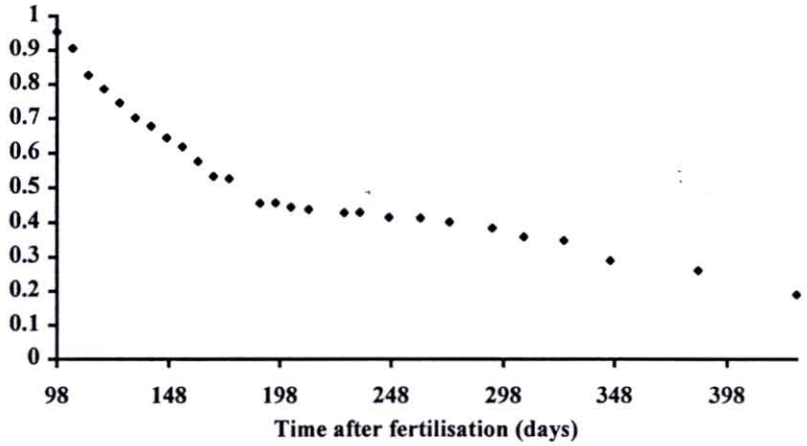


The statistical analysis (Analysis of the covariance, PROC GLM, SAS, 1988) showed that the population affects significantly the growth curve ($p < 0.0001$). The grouping method (Duncan) gave 4 groups significantly different for the growth: (Bonne Anse + Seudre), Port des Barques, PC4 and Arcachon.

The first part of the curve, from day 91 to day 168 is the spat growth recorded in microtanks structure, the second part, from day 168 to day 484, is juvenile and adult growth recorded in 800-liter tanks.

The correlation between the initial total weight measured at day 91 after fertilisation and the total weight at a certain date is given in Figure 2.

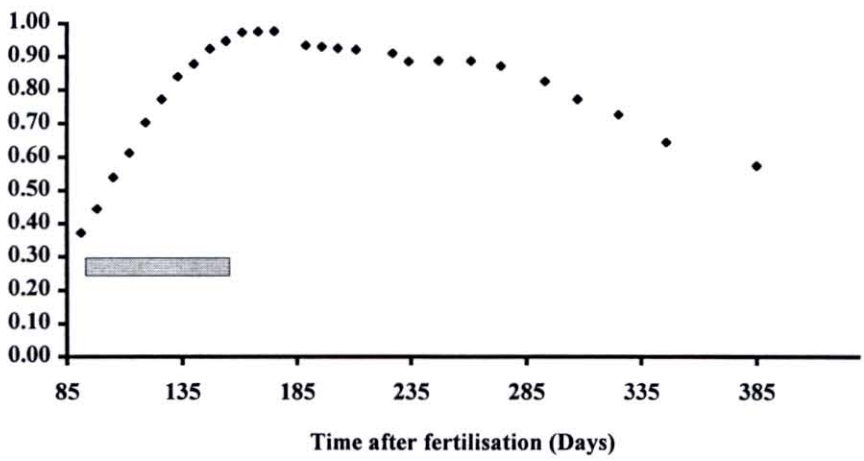
Figure 2: Determination coefficient between the initial total weigth (day 91) and the total weight at the date given in X-axis.



The correlation is decreasing strongly from 0.95 to 0.29. The analysis done per population gave similar results (data not shown).

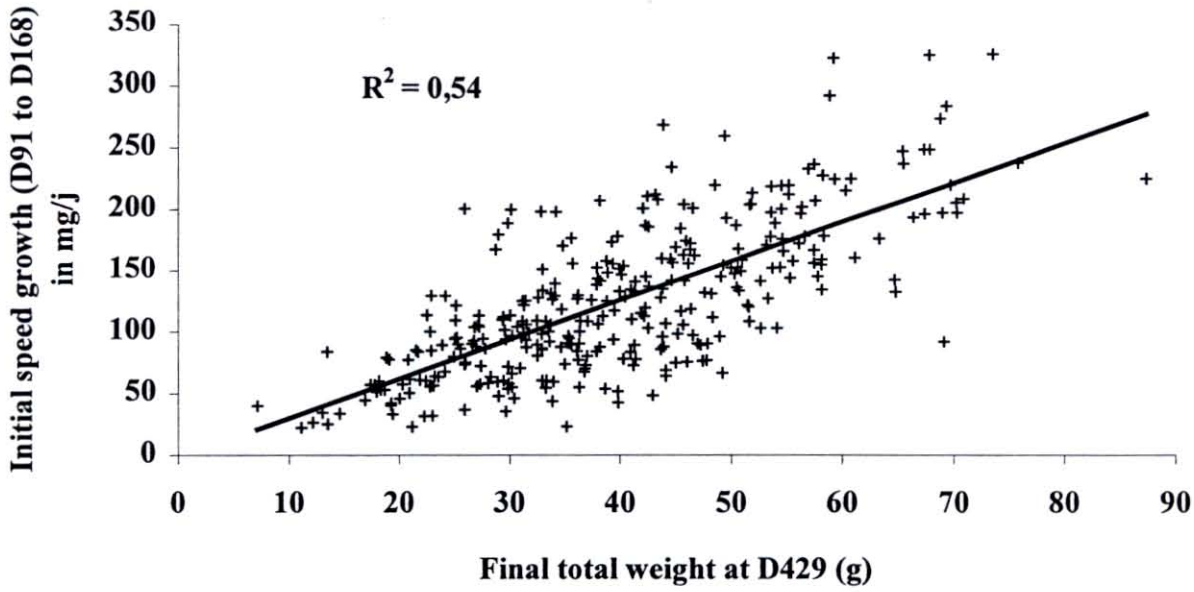
We used a different approach to analyse the correlation in growth between year 1 and year 2. Rather than considering the initial total weight, we calculated an initial growth rate estimated by simple linear regression between day 91 and day 168. An initial growth rate was then computed of each individuals between day 91 and day 168 after fertilisation. The correlation between this initial growth rate and the total weigth at a given date was analysed by linear regression (PROC GLM, SAS 1988). In Figure 3, the coefficient of determination (r square) between the initial growth rate and the total weight at a given date is shown.

Figure 3: Determination coefficient of the correlation between the initial growth rate and the total weight at the date given in the X axis. Horizontal grey bar represents the period of estimation of the initial growth rate.



This time the correlation is decreasing but remained significant and with a r squared of 0.54 at day 429 (Figure 4).

Figure 4: Correlation between the initial growth rate (estimated between day 91 and day 168) and the total weight measured on day 429. This plot corresponds to the last point of Figure 3.



Therefore, 54 % of the total weight measured after 1 year is explained by the initial growth under the conditions of our experiment.

1.4. Conclusion

In conclusion, the total weight is still a good estimator of the growth and is partly determined for a major part by the initial growth. This is consistent with the previous analyses: the 4 pseudocohorts obtained last year showed a difference in length about 1 month after fertilisation. The total weight measured 10 months after showed that this difference was still present and in the same order. It confirms furthermore the results obtained in Bouin hatchery, where animals were reared under different levels of food and recorded individually for growth.

1.5. References

SAS Institute Inc., 1988. SAS/STAT User's Guide, Release 6.03, SAS Institute Inc., 1-1028.

Collet B., Boudry P., Thebault A., Heurtebise S., Morand B. and Gérard A. Relationship netween pre- and post-settlement growth in the Pacific oyster Crassostrea gigas (Thunberg), in prep.

2. *Growth recording in Bouin*

2.1. Introduction

As part of the European program GENEPHYS, teams from Bouin, Palavas and La Tremblade performed an experiment in order to answer to the following questions:

1. Do animals showing different growth rates in their first year (1996) keep these differences during their second year (1997) ?

2. How these growth characteristics are affected by a change in the environment: site of rearing (including all the controlled and uncontrolled parameters) or level of competition for food ?

3. If oysters are reared under 2 contrasted conditions (A = slow growing condition, B = fast growing condition) during a first period, does the relative growth (i.e. with respect to the rest of the individuals reared under the same condition, A or B) or the absolute growth (i.e. with respect to the whole population, whatever is the situation A + B) explain the best the performances observed during a second period ? In other words:
   - Will oysters of similar weight but representative of the biggest animals reared in condition B and of the smallest animals reared in condition A, have a similar growth in their second year ?
   - How is the growth of biggest animals from slow-growing condition and the biggest animals from the fast-growing condition, knowing that they come from the same fraction of the 2 intermediate sub-lots ? Note that the biggest animals in these 2 growing conditions can be genetically different because some "genotype x environment" interaction might exist (the biggest animal in a given environment wouldn't have been necessarily the biggest animal in a different environment).

Therefore, does the weight of an individual at a given age determine its growth potential? In other terms, within a population do the biggest individuals affect more, as competitor for food, small animals rather than bigger animals ? Do the observed differences in growth have a strong enough genetic basis to be maintained when the level of competition between individuals is altered ?

Despite the attempt to split up the phenotypic variability observed into a genetic part and an environmental part (in order to compute an heritability), a trait can be strongly determined both by the environment and a strong genetic basis. This can lead to:

- A strong plasticity of the trait across a range of environmental conditions;
- Differences between individuals, families or populations in a environment as homogeneous as possible. The range of the observed differences depends then on the genetic variability in the study material.

It seems to be the case concerning the growth in oysters. The difficulty here is that

the individuals represent a significant part of the environment itself, because of the competition between individuals, in particular for food. The "common environment" which allow us to compare, for instance, growth families can be tackled in 3 ways:

- at a constant biomass: the amount of food provided is the same for every families but the number of individuals per family varies in such a way that the biomass per family remains constant. Therefore the best families for growth will be represented by a less number of individuals than the other families. As a consequence, the amount of food per individual will be different between the families, which is a bias.

- at a constant number: the amount of food provided is the same for every families but then (again) the amount of food per individual is different between families. Individuals from the best families for growth will get less food (per individual) than those from the other families, which is again, a bias.

- at a constant amount of food per individual: which is of course the best solution but it involves no competition between individuals (one oyster per tank !) or low enough to be insignificant and then to be able to use high amount of food and low densities.

As one oyster per tank is inconceivable, the second solution is often chosen. This solution is satisfactory when the purpose is to compare families in a common environment in order to compute heritability values (knowing that this value can be different in another environment).

As a part of GENEPHYS program, the aim is to precise the ability to grow under condition of limiting amount of food, as it is the case, for instance, in the Marennes-Oleron basin.

2.2. Material & Methods

Available biological material

We have 3 populations called "5 X 5" on which the GENEPHYS program is based on. These G1 populations were obtained from the intra-population crosses of 10 parents (5 females and 5 males) coming from Arcachon, Port-des-Barques and Bonne Anse. This material (G1s) was maintained beforehand in Bouin station. A sample of these 3 populations were recorded individually for their growth (total weight) in La Tremblade station and showed:

- Significant differences between populations but not representative from the origin locations (Arcachon < Bonne Anse < Port-des-Barques),

- Strong differences between individuals (factor higher than 10) which are maintained all along the recording period

The 3 populations "5 X 5" were transferred in two parts from the La Tremblade hatchery to the Bouin nursery: a first part in springtime and then a second part in autumn. Therefore, the history of growth events was different for these 2 parts and we have 2 x 3 = 6 different lots. The table 1 present the material which was available in the Bouin nursery at the beginning of 1997.

Table 1. Oysters available in the Bouin nursery at the beginning of 1997.

Population	Oysters sent from La Tremblade hatchery to Bouin nursery in June 96	Oysters sent from La Tremblade hatchery to Bouin nursery in November 96
Bonne-Anse	1743	2182
Port-des-Barques	1394	2515
Arcachon	849	788

Experimental design

The technical solution selected to provide different level of competition for food consisted in using different densities of "competitors" because it's easier to bring into operation than a daily adjustment of the phytoplankton inflow.

Competitors are 60 g oysters, coming from the same lot, as homogeneous as possible in weight. The mean weight of the competitors was chosen higher than the one of the studied animals (mean total weight of 67.9g).

3 tanks (= 3 "modalities") were settled. The amount of oysters per tank were as follow:

- Tank n°0 = low competition for food: 100 individuals/lot (see sampling hereafter) = 600 individuals individually labelled.
- Tank n°2 = medium competition for food: 100 individuals/lot (see sampling hereafter) = 600 individuals individually labelled + 486 competitors.
- Tank n°4 = high competition for food : 100 individuals/lot (see sampling hereafter) = 600 individuals individually labelled + 1333 competitors.

In every tank, labelled oysters and competitors were mixed together and randomly arranged into 6 racks (i.e. densities of 100, 180, 280 oysters per rack for the tanks n° 1, 2 and 3). Each tank is supplied with sea water and phytoplankton in a same way:

- Sea water : sand filtered, inflow 2.4 m^3/h, temperature 15-16 °C;
- Phytoplankton : daily adjusted by photosynthetic pigments measurements for a 80% of intake in the tank n° 4 (high level of competition for food).

Sampling of the labelled individuals

To maximise the variability in weight of the oysters which were recorded for their growth, the sampling was not performed randomly. For each of the 6 lots, 200 oysters

were previously weighted in order to define 10 classes of total weight per lot. The oysters were next weighed individually till we get up to 30 individuals per class and per lot (i.e. 10 individuals/lot/class/tank), which were labelled individually. As it was not possible to get 30 individuals/lot/class for the extreme weight classes, the initial sampling of the oysters is presented in Table 1. As distribution of weights were different among populations, weight class were also different in order to maximise variability for each lot (se Table 2).

Table 2. Number of labelled oysters per population (Port-des Barques, Bonne anse and Arcachon), date of transfert to Bouin (Autumn 96 = A or Spring 96 = B) and weight class (1 to 10).

			weight class									
Population	Transfert to Bouin	Tank	1	2	3	4	5	6	7	8	9	10
Port-des-	B	1	7	10	10	10	10	10	10	10	10	6
Barques		2	7	10	10	10	10	10	10	10	10	5
		3	6	10	10	10	10	10	10	10	10	6
	A	1	10	10	10	10	10	10	10	9	3	2
		2	10	10	10	10	10	10	10	9	3	2
		3	10	10	10	10	10	10	10	9	3	2
Bonne	B	1	9	10	10	10	10	10	10	10	6	3
Anse		2	10	10	10	10	10	10	10	10	5	3
		3	9	10	10	10	10	10	10	10	5	3
	A	1	10	10	10	10	10	10	10	10	8	5
		2	10	10	10	10	10	10	10	10	7	6
		3	10	10	10	10	10	10	10	10	7	5
Arcachon	B	1	6	10	10	10	10	10	10	10	5	3
		2	5	10	10	10	10	10	10	10	5	3
		3	5	10	10	10	10	10	10	10	5	3
	A	1	7	10	10	10	10	7	3	1	0	1
		2	7	10	10	10	10	7	4	1	0	0
		3	7	10	10	10	10	6	4	1	0	0
TOTAL =	1498											

Growth recording

The labelled oysters were individually weighed 8 times within 3 months (from April 1997 to June 1997). The initial individual total weights are presented in table 3.

Table 3: mean total weight (g) per population and per pre-growing condition

population	Transfer in Bouin in spring (B)	Transfer in Bouin in autumn (A)	mean
Port-des-Barques	21.2	16.4	18.9
Bonne-Anse	22.8	21.9	22.3
Arcachon	19.4	12.0	16.3
mean	21.2	17.5	19.4

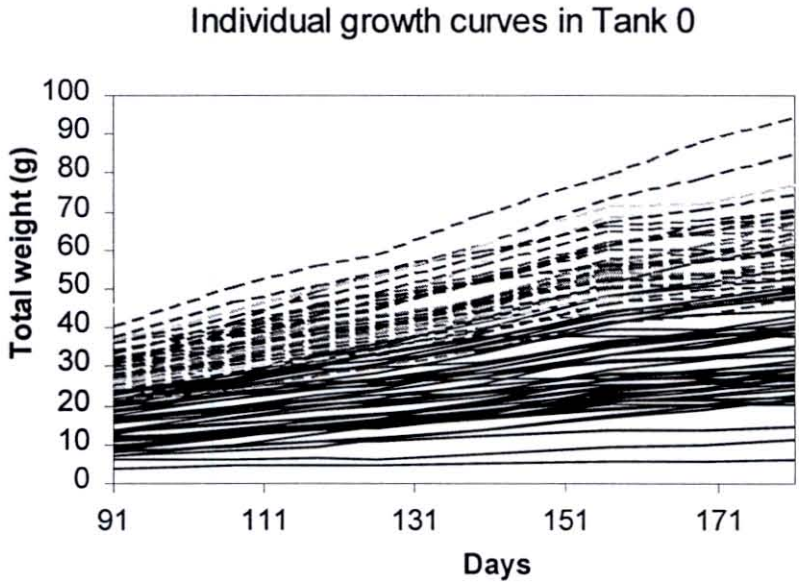
2.3. Results

Individual growth curves

Mortality was very low (2.5 %) during the experiment and therefore did not biased the results in any way. This experience generated 1498 individual growth kinetics. The growth was highly linear as illustrated in Figure 5, which presents the individual growth curves for the oysters monitored in Tank 0. The mean linear regression coefficient value was $r^2 = 0.98$ for each of the 3 experimental tanks. This allowed to calculate the slope of each individual growth curve using linear regression. This slope value is therefore a very good estimate of the growth performance of each oyster during the experiment.

The range variation in growth performances (i.e. slopes) was very broad, as illustrated in Figure 1 for tank 0. The lowest and largest recorded slope were 0.022 and 0.33 (which give a factor 15 between these two extreme values).

Figure 5. Individual growth curves for the oysters monitored in Tank 0.



Differences between tanks

The mean food income was highly similar between tanks (mean values of 21.21, 21.56 and 23.09 µg/l respectively for tanks 0, 2 and 4) (Table 4). The differences in total number of oysters per tank lead to increasing level of food intake between the tanks. This resulted to a mean daily growth rate of 0.24, 0.25 and 0.17 g/d respectively for tanks 0, 2 and 4. We can therefore conclude that food availability was a limiting factor in tank 4 but not in tanks 0 and 2. Consequently, the growth rates measured in tanks 0 (and 2) can be regarded as representative of the growth potential of the labelled oysters.

Table 4. Characteristics of the conditions in tanks 0, 2 and 4 and their impact on growth.

Parameter		Tank 0		Tank 2		Tank 4
Total number of oysters		495	<	976	<	1811
Number of competitors		0	<	486	<	1333
Daily food income/oyster (cells x 10^9)		5.26	<	2.65	<	1.41
Sea water flow (l/h/oyster)		4.93	<	2.51	<	1.36
Food intake measured by the difference in µg/l of photosynthetic pigments :	in	21.21		21.56		23.09
	out	17.38		10.85		6.41
	consumption (%)	18.06		49.67		72.24
Mean growth of the labelled oysters over the 3 months (g/d)		0.24		0.25		0.17

Differences between populations and lots

As recorded in La Tremblade during their first year, the 3 populations exhibited significantly different growth performances (Arcachon having a slowest growth than the two other populations) (table 5). The effect of the pre-growing condition on the growth (i.e. lot within each population) is discussed hereafter.

Table 5: mean slope values (and standard deviation) per population, transfer season and competition level

population	tanks	transfer in Bouin in spring (B)	transfer in Bouin in autumn (A)	mean
Port-des-Barques	0	0.28 (0.12)	0.25 (0.09)	0.27 (0.11)
	2	0.27 (0.10)	0.25 (0.10)	0.26 (0.10)
	4	0.19 (0.07)	0.17 (0.07)	0.18 (0.07)
	all tanks	*0.25 (0.11)*	*0.22 (0.10)*	*0.24 (0.10)*
Bonne-Anse	0	0.32 (0.10)	0.28 (0.10)	0.30 (0.10)
	2	0.29 (0.10)	0.26 (0.10)	0.28 (0.10)
	4	0.20 (0.08)	0.18 (0.07)	0.19 (0.07)
	all tanks	*0.27 (0.10)*	*0.24 (0.10)*	*0.26 (0.10)*
Arcachon	0	0.24 (0.12)	0.19 (0.09)	0.22 (0.11)
	2	0.22 (0.10)	0.18 (0.09)	0.21 (0.10)
	4	0.16 (0.07)	0.12 (0.06)	0.14 (0.07)
	all tanks	*0.21 (0.10)*	*0.16 (0.08)*	*0.19 (0.10)*
mean		0.24 (0.11)	0.22 (0.10)	0.23 (0.11)

Effect of the pre-growing condition on the growth during the experiment

For each population ten classes of initial weight were determined in order to test the effect of the conditions of the first year of growing on the growth during the present experiment.

In the two populations "Ports-des-Barques" and "Arcachon" (for which the two pre-growing conditions had an effect), oysters sampled in the lots transferred in Bouin in autumn (slow pre-growing condition) had a significantly better growth than the ones of similar initial weight but sampled in the lots transferred in spring (fast pre-growing condition) (Figures 6a and 6c). In other words, for a given weight, oysters which are the biggest of a slowed lot grew faster than the smallest of a boosted lot. This tend to support the idea that the expressed growth potential of the oysters maintained in the slow pre-growing environment was under-estimating their genetic potential.

Figure 6a :

effect of the condition during the first year of growing on the second year of growing in the Port-des-Barques population

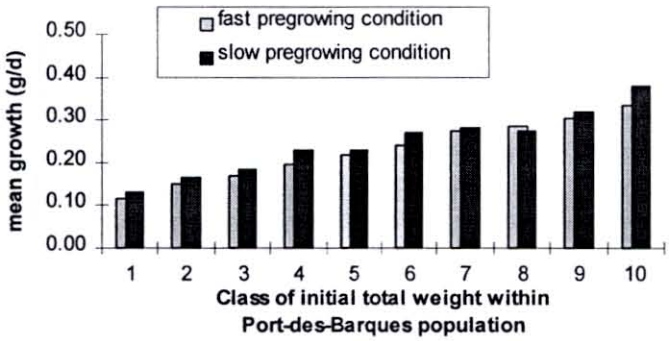


Figure 6b:

effect of the condition during the first year of growing on the second year of growing in the Bonne-Anse population

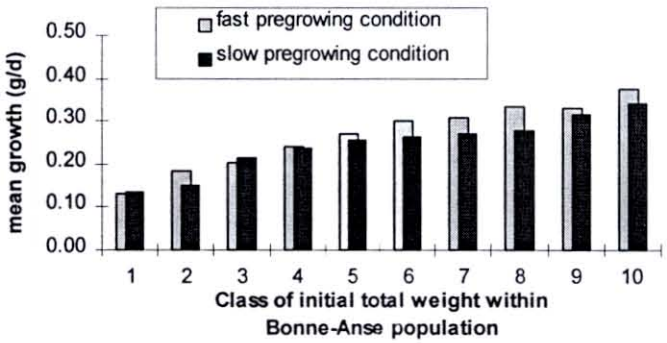
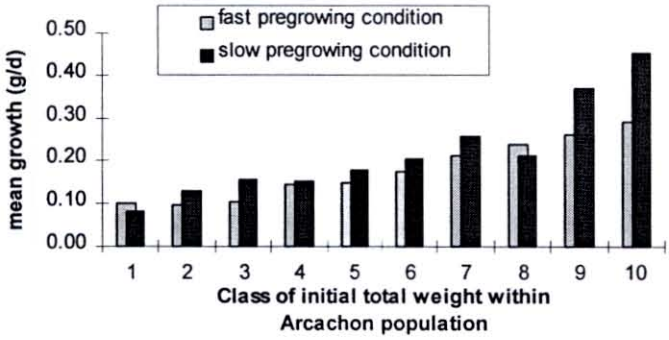


Figure 6c :

effect of the condition during the first year of growing on the second year of growing in the Arcachon population



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n the Bonne Anse population (for which the two pre-growing conditions had no effect), the opposit effect is significant. Up to now, it is very difficult to explain the different behaviour of Bonne Anse population compared with the two others. (Fig6b).

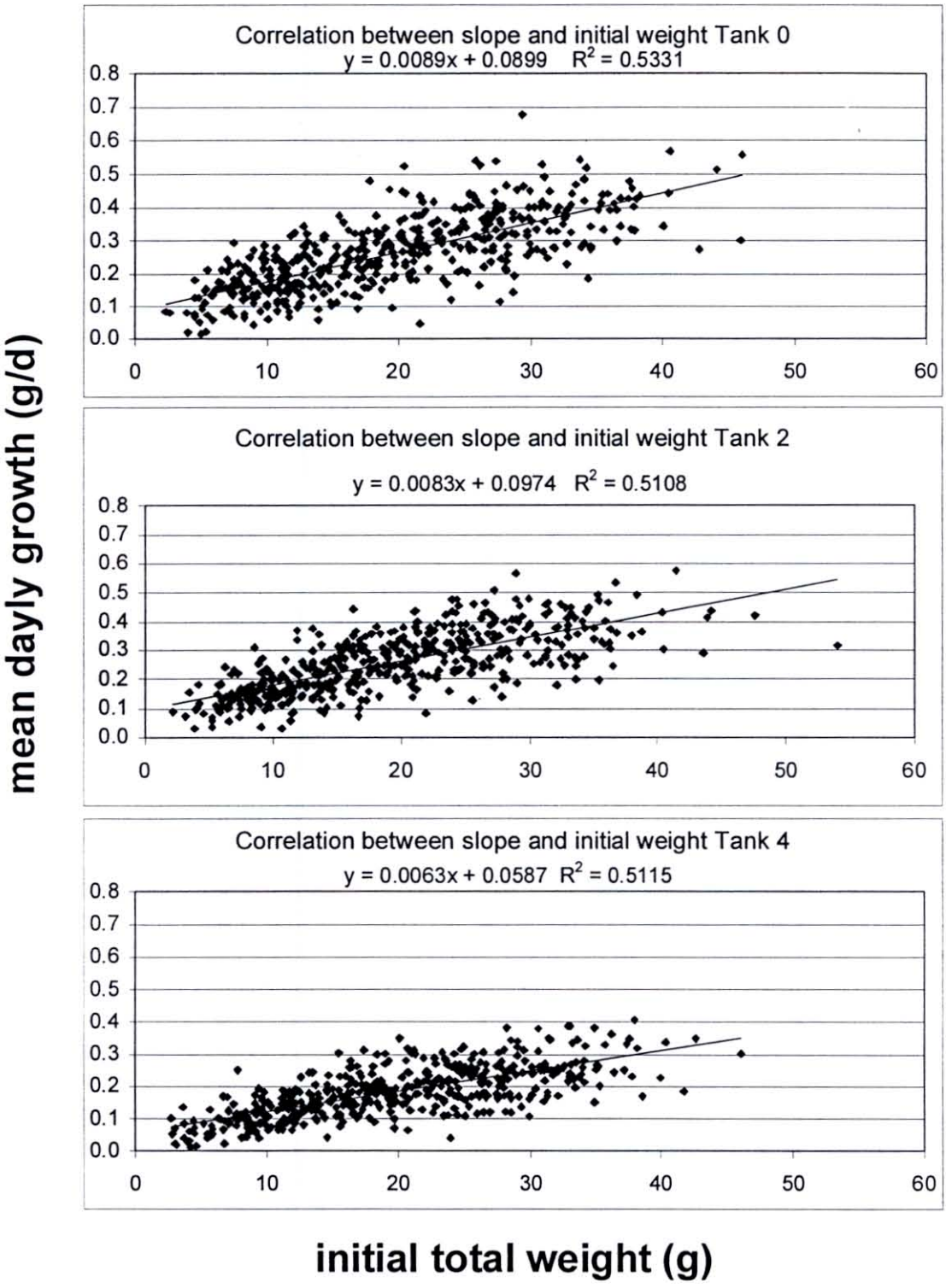
Correlation between initial total weight and growth during the experiment

In order to assess the maintenance of growth performance over time, we plotted the initial total weight with the mean daily growth. (Figure 7) . A good correlation was observed within each tank between these two parameters ($r^2 > 0.51$). This shows that the growth performance is partly conserved over time (first *versus* second year). This correlation can be computed per population and per lot within each population as presented in table 6.

Table 6 : Correlations between initial total weight and mean daily growth per population and per lot.

tanks		Port-des-Barques		Bonne-Anse		Arcachon	
		Transfer in Bouin in spring (B)	Transfer in Bouin in autumn (A)	Transfer in Bouin in spring (B)	Transfer in Bouin in autumn (A)	Transfer in Bouin in spring (B)	Transfer in Bouin in autumn (A)
0	**a**	**0.009**	**0.009**	**0.008**	**0.007**	**0.011**	**0.011**
	b	0.081	0.107	0.135	0.119	0.030	0.055
	r2	*0.50*	*0.48*	*0.53*	*0.55*	*0.45*	*0.53*
2	**a**	**0.008**	**0.010**	**0.008**	**0.006**	**0.009**	**0.013**
	b	0.092	0.092	0.115	0.133	0.044	0.025
	r2	*0.50*	*0.53*	*0.51*	*0.44*	*0.45*	*0.66*
4	**a**	**0.006**	**0.007**	**0.006**	**0.005**	**0.007**	**0.008**
	b	0.064	0.050	0.061	0.079	0.027	0.028
	r2	*0.48*	*0.53*	*0.54*	*0.50*	*0.53*	*0.52*

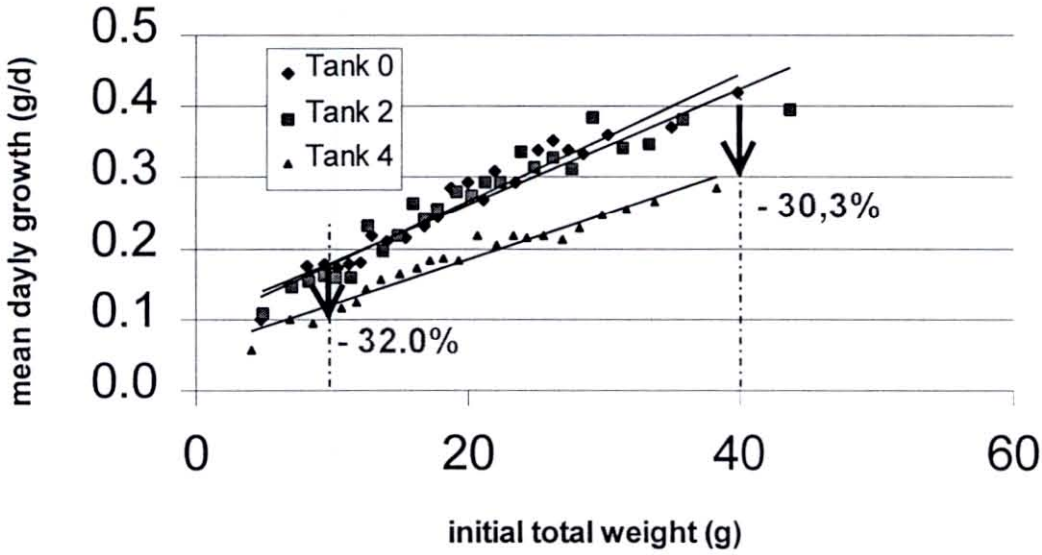
Figure 7. Correlation between the initial total weight and mean daily growth (individual values plotted per tank)



Effect of the competition levels on the relationship between initial total weight and growth during the experiment

Figure 8 allows to compare this relationship among the 3 competition levels. The lowest growth rate of tank 4 is clearly shown by a lower linear regression curve. This competition effect is around 30% and affects similarly large and small oysters. Consequently we can conclude that there is no interaction between competition level and the relationship between initial total weight with the mean daily growth. In other words a lowest food availibility affects on the same way the large and the small oysters. This is the case in our intensive experiemental conditions and it would be of great interest to test this interaction in a broader range of trophic levels (especially lowest food availability or highest competition level).

Figure 8 : effect of competition level (i.e. tank) on the correlation between initial total weight and mean daily growth (each dot represents the mean value for 20 individuals of similar initial total weight).



2.4. Discussion

The data generated by the present experiment is complex to analyse and a detailed statistical analysis still has to be done. But this experiment provides a new results about variability for growth in *Crassostrea gigas*. We demonstrate that growth performance is fairly well ($R^2 \approx 0.5$) maintained over time as the correlation between the weight at the beginning of the experiment (which results from the global growth performance before the experiment) and growth during the experiment. The main remaining question is to know to which extend the observed variability can be explained by competition at the within population level, or if the observed variability has a genetic basis. To answer tat question we plane to set-up a selection experiment for fast growing oysters and test these fast-growing sleeted oysters in the same experimental scheme in 1999. Hopefully, the differences among populations for growth performances observed in La Tremblade and in Bouin suggest that genetic variability exists for growth performances.

In our experiment, tanks 0 and 2 showed similar growth performances. This was not intended at first (as the initial objective was to have an intermediate competition level for food) but this similarity allows us to know that the growth performances observed in tank 0 (and tank 2) are not limited by competition for food. We can therefore assume that the observed performances are somehow the maximum growth performances at the temperature of 16°C. We have here a good estimate of the phenotypic value of growth performance for each labelled oyster.

One very interesting results is the apparent absence of interaction between competition level (i.e. tank) and the relationship between initial total weight and slope of growth. This might be due to the fact that, even in the high competition tank, growing conditions were still fairly intensive, and therefore not representative of field growing conditions in over-exploited sites like Marenne-Oléron estuary in France.

3. Growth recording in Palavas

Reception on 8 April 1997 of 6 batches of juvenile oysters corresponding to different GENEPHYS populations:

Batches	**Ronce 2**	**Ronce 3**	**Ronce 4**	**Bouin 2**	**Bouin 3**	**Bouin 4**
Populations	Port des Barques (Ronce*)	Bonne Anse (Ronce)	Arcachon (Ronce)	Port des Barques (Bouin)	Bonne Anse (Bouin)	Arcachon (Bouin)
Numbers	1650	1595	199	1360	1095	560

**Nursed in Ronce*

Weight frequency histograms of each populations has been done to design an experiment allowing growth comparisons among different homogeneous weight classes inside populations and between populations.

The rearing unit is a rope with 30 rows of two cemented oysters making 60 oysters per unit. Weight classes were chosen for being common to a maximum of batches with possibility of making a maximum of replicates for each weight class. The variability of the total numbers and sizes of oysters in the different batches were limiting factors.

Three batches (Bouin 2, Bouin 3 et Ronce 3) were divided in four common weight classes (<16g - 16 à 20g - 20 à 24g - >24g) with four replicates per weight class (four ropes of 60 oysters), making an experiment with 2 factors: populations (three levels) and weight classes (four levels). These batches will allow comparisons intra and inter populations. The other batches were divided in different ways allowing only comparisons intra population. Characteristics of the different batches are given in the following tables.

ANNEX III
Individual Progress Report
Participant n°1

Weight classes	Number	Mean weight (g)	Standard deviation	Rope number
BOUIN 2				
	60	12.9	2.0	38
0 to < 16	60	13.0	1.9	40
	60	12.9	1.8	41
	60	13.0	1.9	42
	60	17.6	1.0	43
16 to < 20	60	17.8	1.1	44
	60	17.9	1.0	45
	60	18.0	1.1	46
	60	21.5	1.1	47
20 to < 24	60	21.8	1.2	48
	60	21.6	1.1	49
	60	22.1	1.3	50
	60	26.8	2.4	51
> to 24	60	26.9	2.8	52
	60	27.1	2.4	53
BOUIN 3				
	60	13.4	1.9	73
< to 16	60	13.2	1.8	74
	60	12.7	2.0	75
	60	13.0	1.6	76
	60	17.2	1.1	77
16 to < 20	60	17.1	1.2	78
	60	17.9	1.0	79
	60	18.0	1.1	80
	60	21.5	1.2	81
20 to < 24	60	21.5	1.1	82
	60	21.7	1.1	83
	60	21.4	1.1	84
	60	26.1	1.9	85
> 24	60	25.6	1.9	86
	60	27.8	3.6	87
	39	27.1	3.2	88
BOUIN 4				
	60	13.5	2.0	90
< to 16	60	13.4	1.6	91
	60	13.6	1.9	92
	60	17.4	0.9	93
16 to < 19	60	17.5	0.8	94
	35	17.5	1.0	95
	60	22.3	2.4	96
> to 19	60	21.7	2.0	97

Weight classes	Number	Mean weight (g)	Standard deviation	Rope number
RONCE 2				
	60	8.2	1.4	10
5 to < 10	60	7.6	1.4	11
	60	7.4	1.1	12
	60	7.5	1.1	13
	60	11.1	0.8	14
10 to < 13	60	11.4	0.8	15
	60	11.3	0.8	16
	60	11.3	0.8	17
	60	14.5	0.9	24
13 to < 17	60	14.8	1.3	25
	60	14.8	1.2	26
	60	14.7	1.2	27
	60	19.3	2	28
17 to < 24	60	19.1	1.8	29
	60	19.3	2.2	36
	60	19.9	2.1	37
RONCE 3				
	60	11.2	2.5	54
< to 16	60	12.0	2.3	55
	60	11.8	2.5	56
	60	11.5	2.4	57
	60	17.6	1.1	59
16 to < 20	60	17.8	1.2	60
	60	17.9	1.1	61
	60	17.9	1.2	62
	60	21.7	1.1	63
20 to < 24	60	21.6	1	64
	60	21.7	1.1	65
	60	22.1	1.1	66
	60	27.6	3.5	67
> to 24	60	27.6	3	68
	60	27.2	2.6	69
	60	27.0	2.8	70
RONCE 4				
5 to < 9	60	6.8	0.9	98
9 to < 11	60	9.7	0.6	99
> to 11	48	12.6	1.3	100

A total of 74 ropes have been randomly placed (except for Ronce 4 and Bouin 4) on the IFREMER rearing table, in the Marseillan area of Thau between 8 and 11 April 1997. Due to a dystrophic event during summer in this area, we have had to move the ropes on 9 September to another area of Thau near Bouzigues (see chart). This movement caused a loss of about one hundred oysters which were randomly detached from some ropes.

The rearing system with cemented oysters on ropes does not permit weighing. The results will be available only at the end of experiment in December. The last visit in October showed a very good growth with most oysters apparently reaching a commercial size.

Task 4 Physiological analyses

Sub-task 4.1 : Temporal stability analysis of physiological traits,

1. Introduction

The aim of this sub-task is to study an eventual time stability of the physiological performances for individuals of a same age class.

Last year, it was concluded that individual scope for growth measured classically (discrete samples) was not a good predictor of individual growth. It was decided to develop continuous monitoring clearance rate and oxygen consumption in order to improve the precision of all physiological estimations, taking into account the level of activity of the oysters.

Such experiments were achieved in April, September, October and December 1997. In April, the automatic sampler for filtration rate was not working, only continuous oxygen consumption was recorded and data were included in a publication (Bougrier et al., 1998). Data from that last experiment are not yet treated.

2. Material and methods

Material. Intrapopulation crosses were carried out in April 1996 from 3 natural populations collected along the French west coast: Arcachon bay (ARC), Bonne Anse Bay (BA) and Port des Barques (PB). 30 labelled oysters (reared in the same environment from their birth and their Individual growth recorded from 3 months after fertilisation) were selected for that sub-task according to their total weight in March. As measurements, on the same animal, must be repeated all along the year, oysters must not be killed after experiment. Non labelled oysters were reared in the same environment in order to insure the determination of dry weight of studied animals by a relationship between dry tissue weight (DW) and total weight (TW) established from 30 non-labelled animals. Some mortalities occurred in May and June. It was decided to reared these animals in a recirculating system at constant temperature (representative of natural seawater temperature) and fed *Skeletonema costatum*. That constant temperature was 20°C from July to September, 18° in October, 14 °C from November to December, and will be 10°C from January to February and 14°C from March to April.

Continuous oxygen consumption monitoring. The monitoring system, based on a recirculating system, is described complety in Bougrier et al. (1998). The following description is numbered according to the diagram in Fig. 1:

1) Seawater was stocked in a tank at constant volume.

2) Seawater was injected into the system at a flow rate of 15 to 30 ml/mn according to the size of the oyster, using a peristaltic pump.

3) Dissolved oxygen measurement requires the absence of gaseous bubbles in seawater. A chamber for the elimination of air bubbles was therefore incorporated in the circuit.

4) The flow of the water through the measurement chamber was controlled using a flowmeter.

5) The measurement chamber was placed in a water bath in order to keep temperature constant.

6) An oxymeter probe was fixed on the top of the chamber. Signals were collected on paper recorders or by a microcomputer using an acquisition card.

Continuous measurements were carried out over a 24 h period for each oyster.

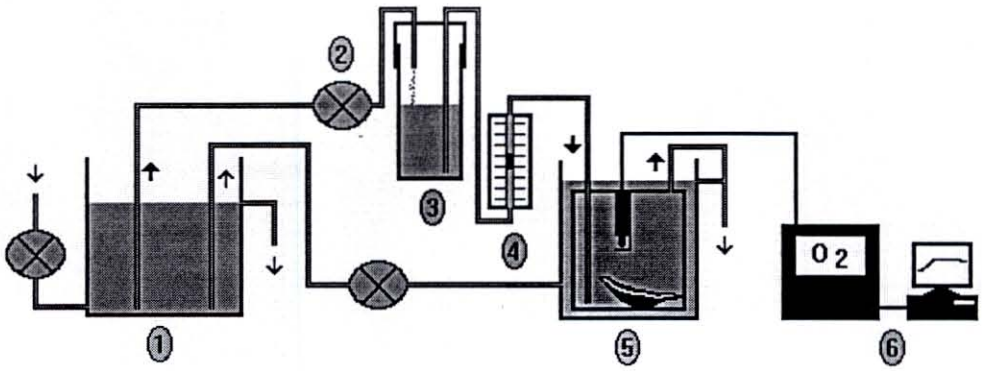


Fig. 1: Continuous oxygen consumption monitoring system. (Details in text)

An active period was characterised by a duration (t, Fig.2) starting with a rapid decrease of oxygen concentration (left arrow) and stopping with a relatively slow decrease of oxygen in the chamber.

Respiratory Time Activity (RTA) was defined as the proportion of time spent active (Fig.2), equal to the sum of active period duration (t1 +t2 +.... +tn) divided by the total recording time (24 h).

The oxygen consumption was estimated as the difference between oxygen concentration at the inflow and the outflow of the chamber taking into account flowrate and time.

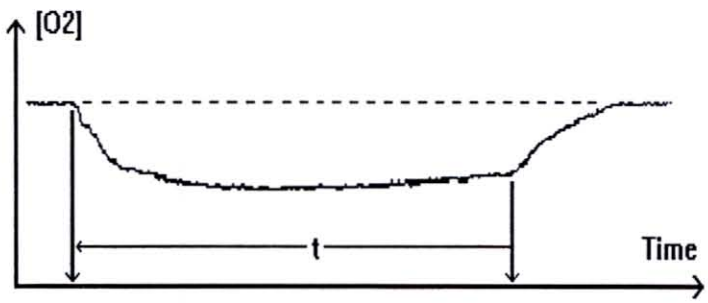


Fig. 2: Example of continuous oxygen concentration recording. An active period was characterised by a duration (t) starting with a rapid decrease of oxygen (left arrow) and stopping with a relatively slow decrease of oxygen (right arrow).

Continuous filtration rate monitoring. A automatic sampler was included to our classical flowthrough system (Fig. 3).

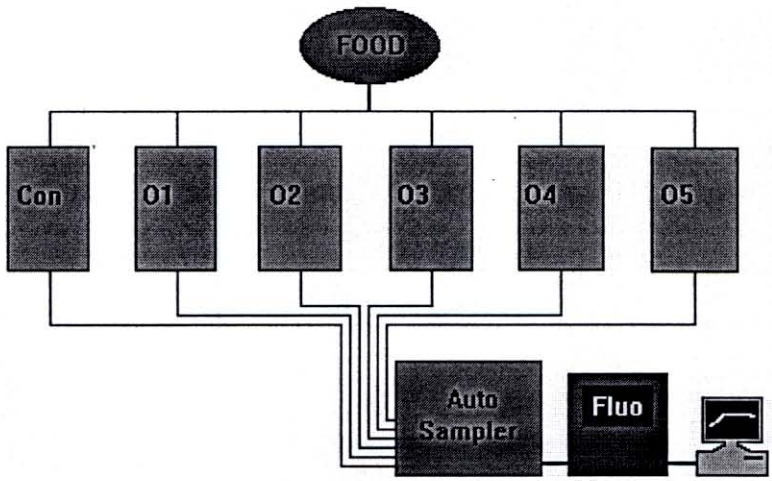


Fig.3: Continuous filtration rate monitoring system.

By limiting manipulation, automation allows to speed up the sampling procedures and data handling and warrants that samples are obtained and processed in identical conditions with the lesser risk of contamination.

The sampling device: Using a micro-gear pump, water samples are sequentially drawn from each experimental chamber via polyamide tubes (Rilsan, 4mm id). Using CPV, 24V DC solenoid valves, these samples are conveyed through a single water outlet, to the measuring device (fluorometer), before being returned to the rearing tank. The speed of the pump is adjusted to allow stable signals from the measuring instrument. The valves are controlled through a multirelay card connected to a computer or they are driven by an programmable industrial process controller. The computer also store the data after they are digitised via an analogue/digital card-converter. The pump and the valves are sheltered in a watertight PVC box. The number of channel to monitor and the characteristics of the sampling cycle are set using a specially designed software. The various stages of the sampling sequence are the following: (1) the first valve is opened, (2) the corresponding tubing is rinsed at high water flow, (3) the speed of the pump is decreased during data acquisition, (4) the pump is stopped, (5) the valve is closed, (6) the next valve is opened...

In order to take into account the response time of the flurometer, only records from the last 30 seconds of the sampling periods are considered in later analysis.

In our experiment, during the first minute, the outflow of the control chamber (Con, Fig. 3) is measured, then during the second minute it was the outflow of oyster 1 (O1, Fig. 3), during the seventh minute, the outflow of the control was measured. That meaned, that an oyster is analysed during 1 minute each 6 minutes. An active period was characterised by a positive retention of the animal ($r > 1\%$), estimated by the difference of food concentration at the control and studied animal chambers multiply par 100.

Firstly, Feeding Time Activity (FTA) was defined, as RTA, as the proportion of time spent active for feeding processes (positive retention) during the 24 h total recording time (Fig. 4).

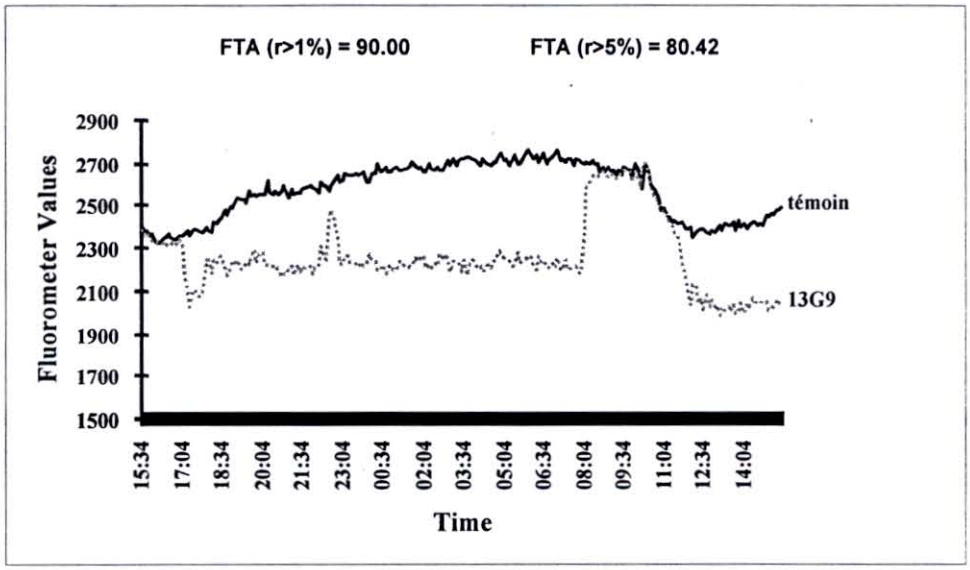


Fig.4 : Example of continuous filtration rate monitoring. (Témoin: control, 13G9: studied animal)

However, it was noticed, for some oysters, values of FTA relatively important while the animal did not produced biodeposits at all. That could be explained by the relatively bad precision of the fluorometer. So, FTA was finally defined, as the proportion of time spent active, with a retention value upper than 5%, during the 24 h total recording time.

The filtration rate was estimated as the difference between food value at the outflow of the control chamber and the studied animal chamber taking into account flowrate and time.

Water and biodeposits samples. 5 to 8 water samples for chlorophyll and Particulate organic matter (POM) were collected each day in order to calibrate the fluorometer. Pseudofaeces and faeces were collected separately 5 times per day in order to estimate their daily production (POM). Estimation of physiological parameters were determined using classical calculations.

3. Preliminary results and discussion

Activities. In April, only RTA was recorded. The mean value of RTA was 86%, meaning that an oysters which was immersed for 24h, consumed oxygen only about 20 h. Such results were in agreement with others (old) experiments and were included in a paper (Bougrier et al. 1998). These authors observed that the mean RTA in normal condition was about 85%, and noticed for different experiments RTA value of about 60%. Such lower values were explained by a possible post-spawning condition. It was noticed RTA values of 65% and 62% during September and October experiment, respectively. The observation of the non labelled oysters used for the estimation of dry weight showed that they were not in a post-spawning condition Such results seemed to

be in contradiction with Bougrier et al. (1998), and at present, there is no explanation for these low values.

FTA values for September and October experiment were 43% and 69%, respectively. As, it was the first time that such experiment occurred, it was difficult to explain those values. However, such results seemed to be i.)in agreement with results of Galtsoff (1964) who observed an activity of 71%, ii.) in disagreement with those of Loosanoff and Nomejko (1946) and Higgins (1980) who observed about 95%. But such results were obtained by the estimation of valve activity.

As for RTA (Fig. 5), it was not noticed some relationship between FTA (Fig. 5) and the total or dry weight of the animals .

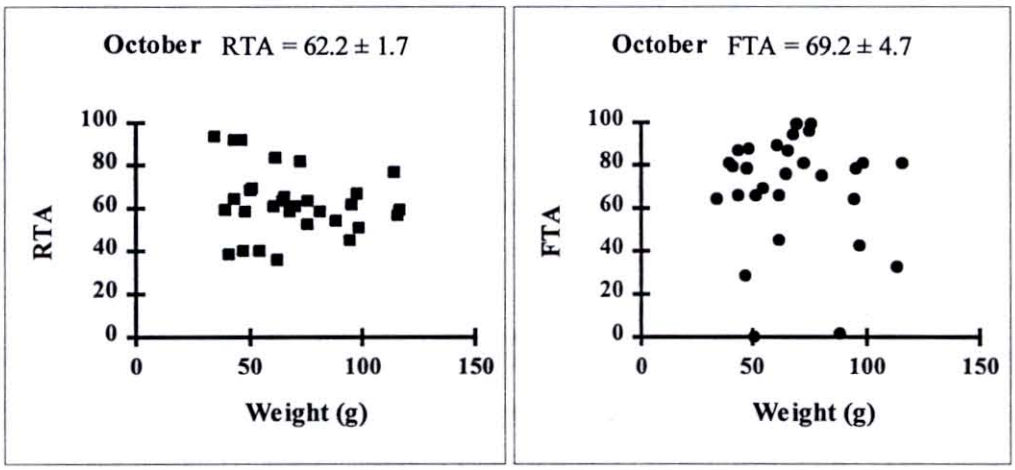


Fig. 5: RTA (left) and FTA (right) value fitted to total weight.

Feeding processes: Results for all physiological parameters seemed to be in contradiction from an experiment to the other. For example, if we considered the organic absorption rate (OAR), it was noticed in September, for highly active oysters, a negative relationship between AOR and total weight, while this relationship could be positive in October experiment (Fig. 6).

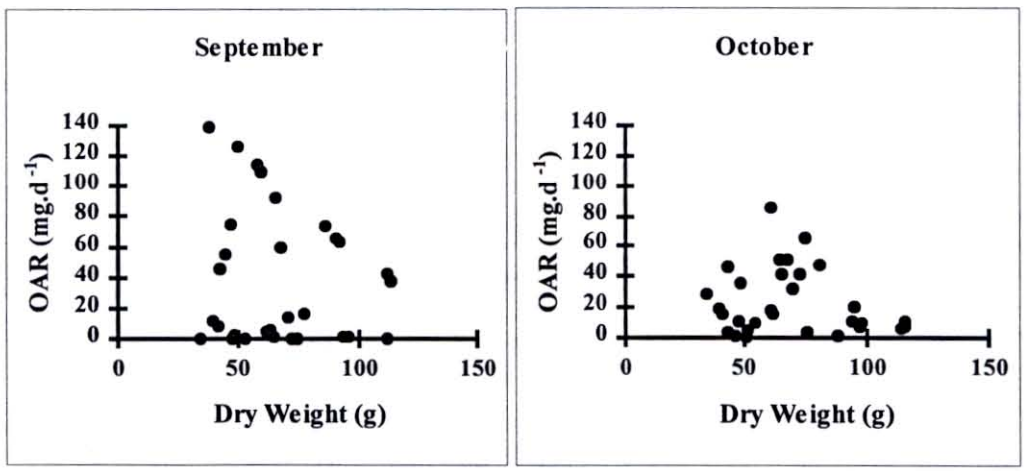


Fig. 6: Organic absorption rate fitted to total weight.

However, it was noticed, despite a large variability, that a linear positive relationship between i.) oxygen consumption rate and RTA (Fig. 7), and ii.) organic filtration rate and FTA (Fig. 8), could be established. For September OFR data, a better fitting would be obtained using a non linear relationship.

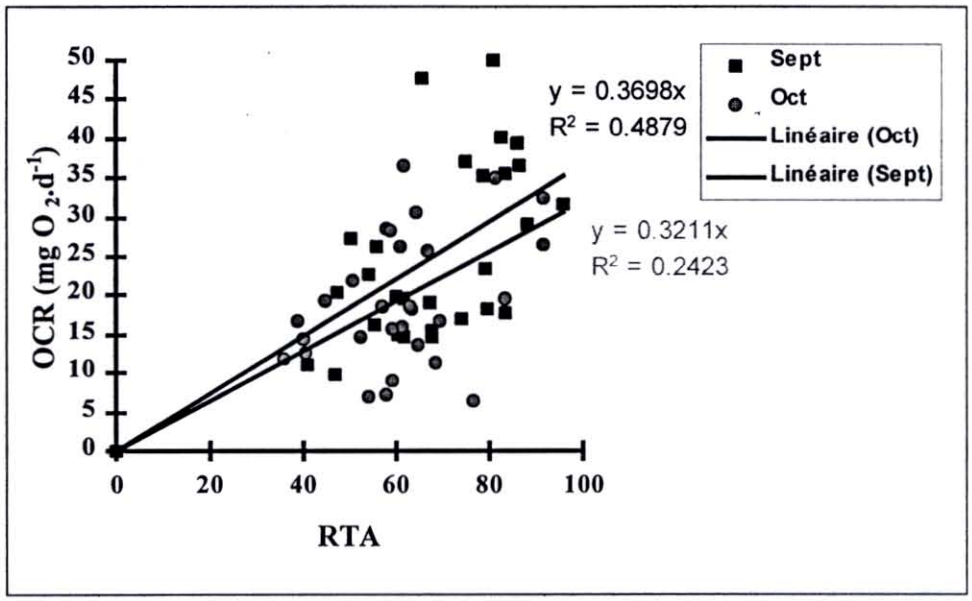


Fig. 7: Relationship between oxygen consumption rate and respiratory time activity.

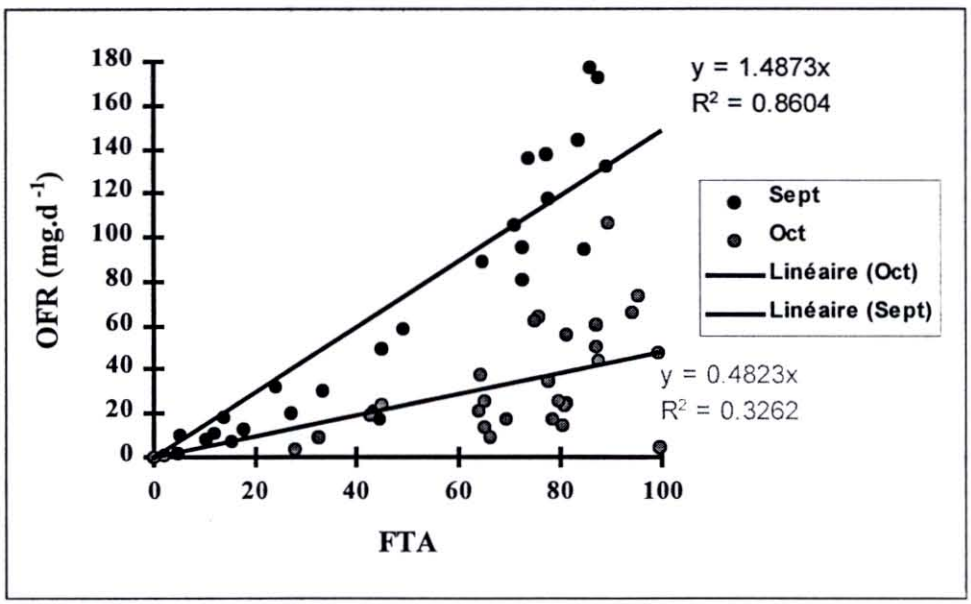


Fig. 8: Relationship between organic filtration rate and feeding time activity.

Temporal stability of scope for growth: Individuals scope for growth value measured in September and in October were fitted Fig. 9. It was noticed that most of the oysters which SFG value were high in September presented very low or negative values in October. On the opposite, oysters characterised by a low value in September presented high value in October. At present, it could be conclused, there is no temporal stability of scope for growth.

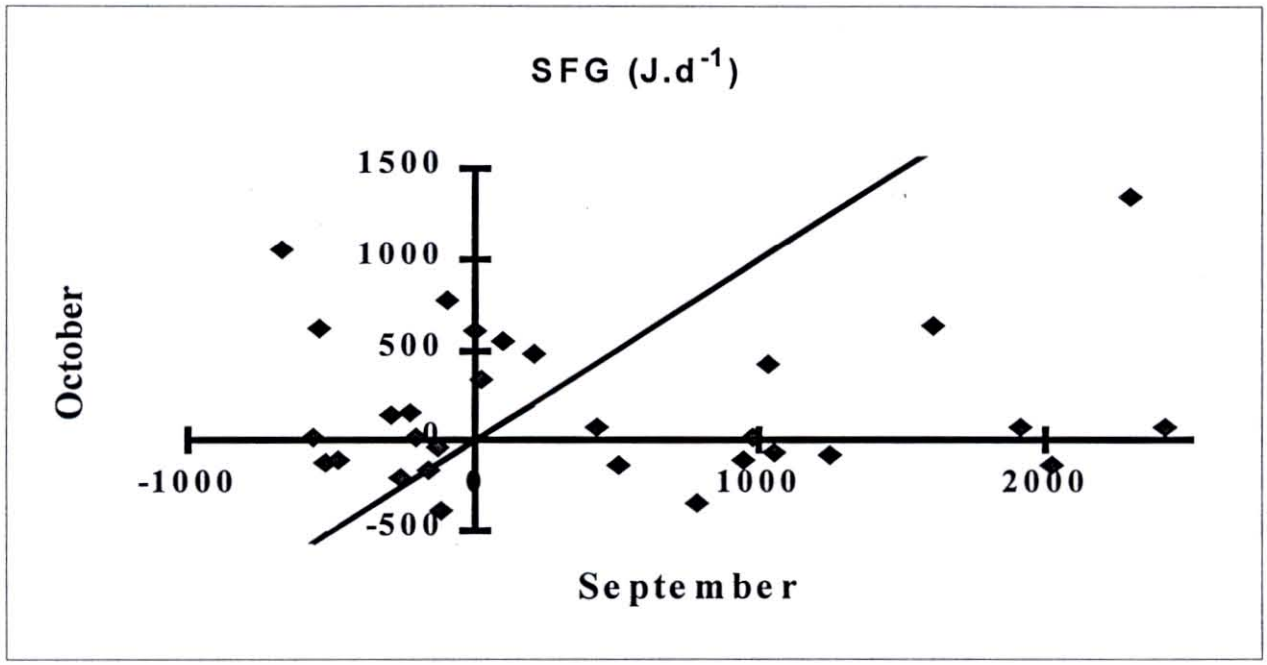


Fig. 9: October scope for growth fitted to September scope for growth.

At present, preliminary results seem confused. New results from the other three experiments would improve the understanding of the temporal stability of physiological performances of G1 animals.

4. References

Bougrier S., Collet B., Geairon P., Geffard O., Héral M., Deslous-Paoli J.M. (1998) Respiratory time activity of the Japanese oyster Crassostrea gigas (Thunberg). J. Exp. Mar. Biol. Ecol., 219: 205-216.

Galtsoff P.S. (1964). The american oyster, *Crassostrea virginica* Gmelin. *Fish. Bull. US.*, **64**: 1-480.

Higgins P.J. (1980). Effect of food availability on the valve movements and feeding behavior of juvenile *Crassostrea virginica* (Gmelin). I. Valve movements and periodic activity. *J. Exp. Mar. Biol. Ecol.,* **45**: 229-244.

Loosanoff V.L., Nomejko C.A. (1946). Feeding of oyster in relation to tidal stages and the periods of light and darkness. *Biol. Bull. Mar. Biol. Lab. Woods Hole (Mass.),* **90**: 244-264.

Sub-task 4.2 : Non destructive measurements of the physiological functions,

1. Introduction

According to the last report (1996), animals from three intrapopulation for analysed for i.) non destructive physiology and then ii.) destructive physiology and genetical analyses.

Animals were acclimated 2 weeks to low food availability and a temperature of 15°C. At the end of this first experiment (non destructive physiology, June 1997), Partner 2 realised the protein turn-over (sub-task 4.3) experiment. Then animals were dissected and sub samples were prepared for physiological (enzyme and proteolitic analyses) and genetical (microsatelites and allozymes) analyses.

2. Material and methods

Intrapopulation crosses were carried out in April 1996 from 3 natural populations collected along the French west coast: Arcachon bay (ARC), Bonne Anse Bay (BA) and Port des Barques (PB). 68 labelled animals were drawn from these populations with a total weight ranging from 9.13 g to 71.12 g. Individual growth was recorded from 3 months after fertilisation to the experiment (June 1997). As tissues of those animals were used for genetical (microsatellites and allozymes) and physiological (enzyme and proteolitic activities) analyses, dry tissue weight was estimated by a regression relationship between dry tissue weight (DW) and total weight (TW) established from 30 non-labelled animals of each population.

The animals were acclimated 2 weeks to the experimental conditions: constant seawater temperature (15 °C) and fed *Skeletonema costatum* at a low enough Total Particulate Matter (TPM of about 2 mh.l^{-1}) to not induce pseudofaeces production. Physiological parameters were determined in June 1997 before spawning in a flow-through system described by Anonymous (1987), using the biodeposit method according to Urrutia et al. (1996) and Hawkins et al. (1996).

Water samples collected from the outflow of an empty (control) tray were filtered onto ashed and pre-weighted 47 mm GF/C filters (Watman), rinsed with distilled water, dried in an oven (60 ° C for 24h), weight and then ashed in a muffle furnace (450 ° C for 4h) before final weighting. In this way Total Particulate Matter (TPM, mg.l^{-1}) and Particulate Inorganic Matter (PIM, mg.l^{-1}) were determined, and the Particulate Organic Matter (POM, mg.l^{-1}) was estimated as the difference between TPM and PIM.

Faeces produced by each animal were collected and treated as described for the water samples. No pseudofaeces was produced as TPM values were low enough to not induce animal rejection. The total (ER, mg.h-1), inorganic (IER, mg.h-1) and organic (OER, mg.h-1) Egestion rates were determined.

Assuming that inorganic material is not absorbed through the digestive tube, IRR + IER

was considered as the inorganic filtration rate (IFR, $mg.h^{-1}$), clearance rate was estimated as CR ($l.h^{-1}$) = IFR / PIM. Filtration rate was calculated as FR ($mg.h^{-1}$) = CR x TPM, and organic filtration rate as OFR ($mg.h^{-1}$) = CR x POM. Ingestion rates of total and organic particulate matter were estimated as IR ($mg.h^{-1}$) = FR and OIR ($mg.h^{-1}$) = OFR, respectively, and then total and organic absorption rates were calculated as AR ($mg.h^{-1}$) = IR - ER and OAR ($mg.h^{-1}$) = OIR - OER. Absorption efficiency was defined as the ratio of absorbed and ingested organic material (Ae = OAR / OIR).

The rate of oxygen consumption (OCR, mg $O_2.h^{-1}$) was measured in a closed chamber of 350-1000 ml, according to the size of each animal. OCR was estimated as the rate of decrease oxygen concentration inside the measurement chamber, as recorded by oxymeter probes (Orbispheres Laboratories).

Finally scope for growth was estimated as SFG ($J.h^{-1}$) = A - R, were A and R were, absorption and oxygen consumption respectively, expressed in term of energy:

A ($J.h^{-1}$) = AOR x 20.132

R ($J.h^{-1}$) = OCR x 14.147, energy conversion factors for absorption and oxygen consumption were from Whyte (1987) and Bayne et al., (1985), respectively.

All the statistical analyses were computed using SAS software (SAS Institute, 1988). Analyses of variance, analyses of covariance and regressions were made by using General Linear Model procedure.

3. Preliminary results

The total weight of studied animals ranged from 9.13 g to 71.12 g (Fig. 1).

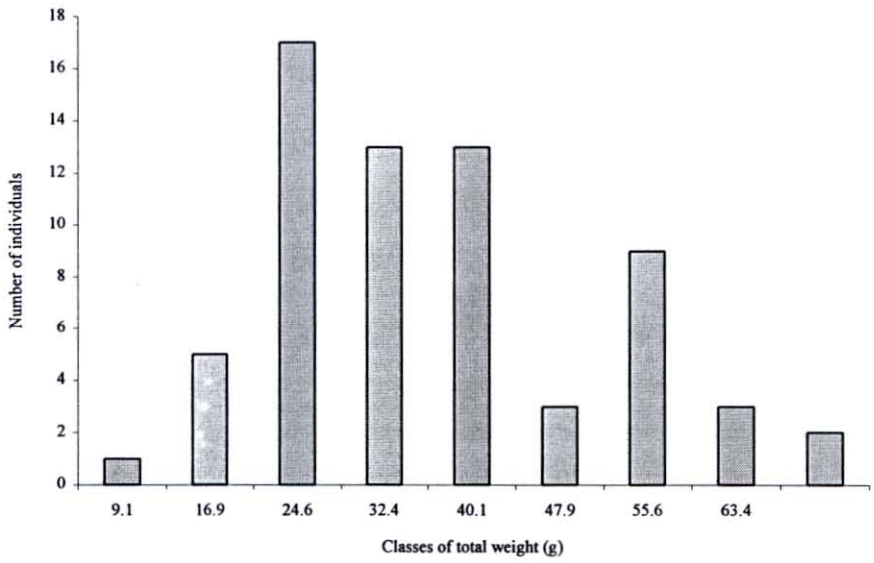


Fig. 1 Distribution in the total weight (g) of the studied animals.

The growth rate for each individuals from the 91st to the 147th day after fertilisation estimated by regression analyses ranged from 1.7 mg.day^{-1} to 16.9 mg.day^{-1}(Fig. 2).

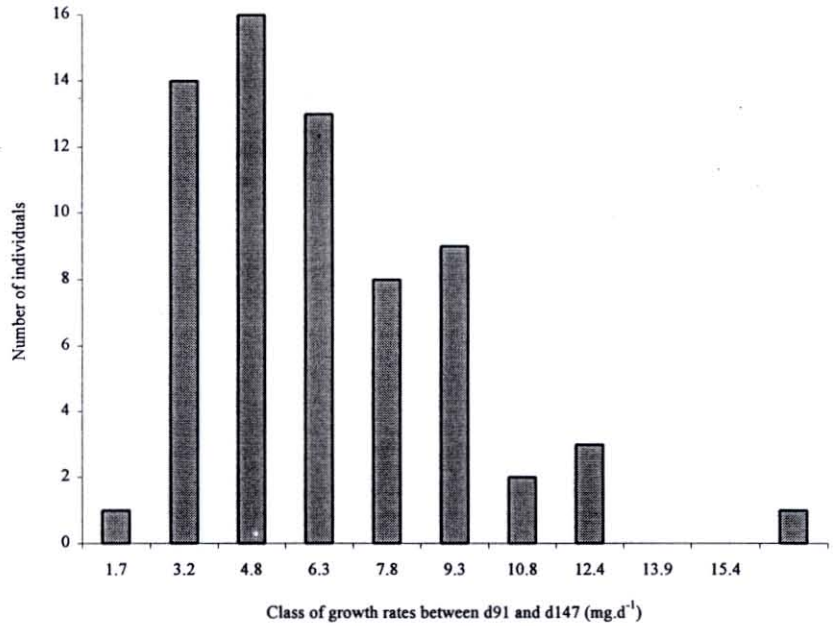


Fig. 2 Distribution in the initial growth rates (mg per day) of the studied animals.

The linear relationship between the total weight and the dry weight is shown in fig. 3.

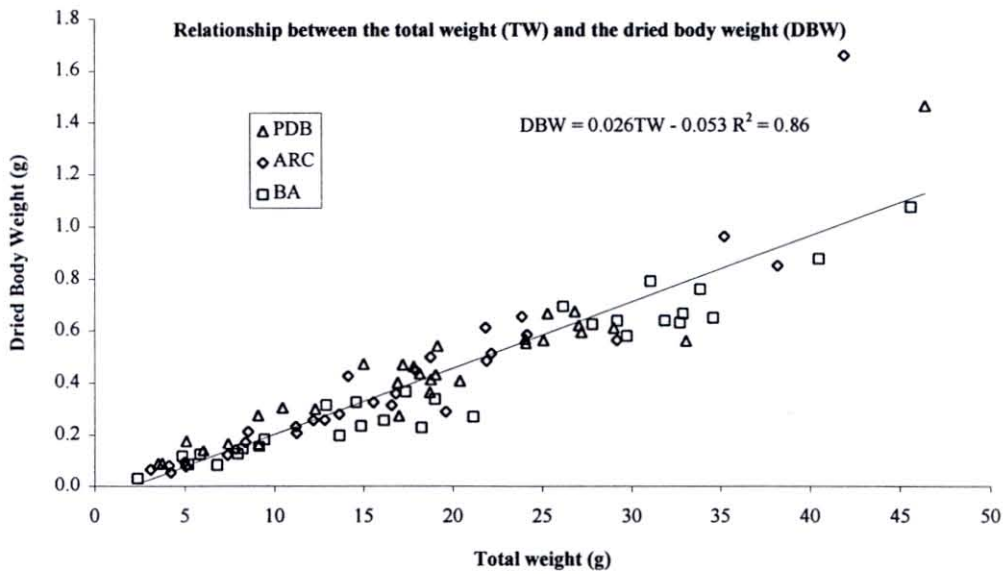


Fig. 3 Relationship between the dry weight (DW) and the total weight (TW) used for the estimation of the dry weight for the population of Port des Barques (PDB), Arcachon (ARC) and Bonne Anse (BA).

The table 1 shows the components of the energetic budget. Despite significant dry weight ($p = 0.02$), no significant differences between the population were found.

	Arcachon	Bonne Anse	Port des Barques
DW (g)	**0.61 ± 0.02**	**0.89 ± 0.02**	**0.86 ± 0.01**
TPM (mg/l)	2.01 ± 0.08	2.01 ± 0.08	1.87 ± 0.06
OC (ratio)	0.42 ± 0.01	0.41 ± 0.01	0.41 ± 0.01
CR (l/h)	1.08 ± 0.03	1.35 ± 0.05	1.17 ± 0.03
OIR =OFR (mg/h)	0.71 ± 0.02	0.88 ± 0.03	0.80 ± 0.03
OER (mg/h)	0.37 ± 0.01	0.44 ± 0.01	0.45 ± 0.01
OAR (mg/h)	0.33 ± 0.01	0.46 ± 0.02	0.35 ± 0.01
Ae (ratio)	0.27 ± 0.03	0.33 ± 0.03	0.27 ± 0.03
OCR (mg/h)	0.86 ± 0.02	1.03 ± 0.02	0.93 ± 0.01
SFG (J/h)	-4.46 ± 0.34	-4.34 ± 0.53	-5.46 ± 0.37

Table 1. Mean (± standard error) of the physiological parameters of the three populations(DW: dry weight, TPM: Total particulate matter, OC: organic content, OFR: organic filtration rate, OIR: organic ingestion rate, OER: organic egestion rate, OAR: organic absorption rate, Ae: absorption efficiency, OCR: oxygen consumption rate and SFG: Scope for growth).

Significant linear relationship between oxygen consumption rate and dry weight (Fig. 4) was noticed.

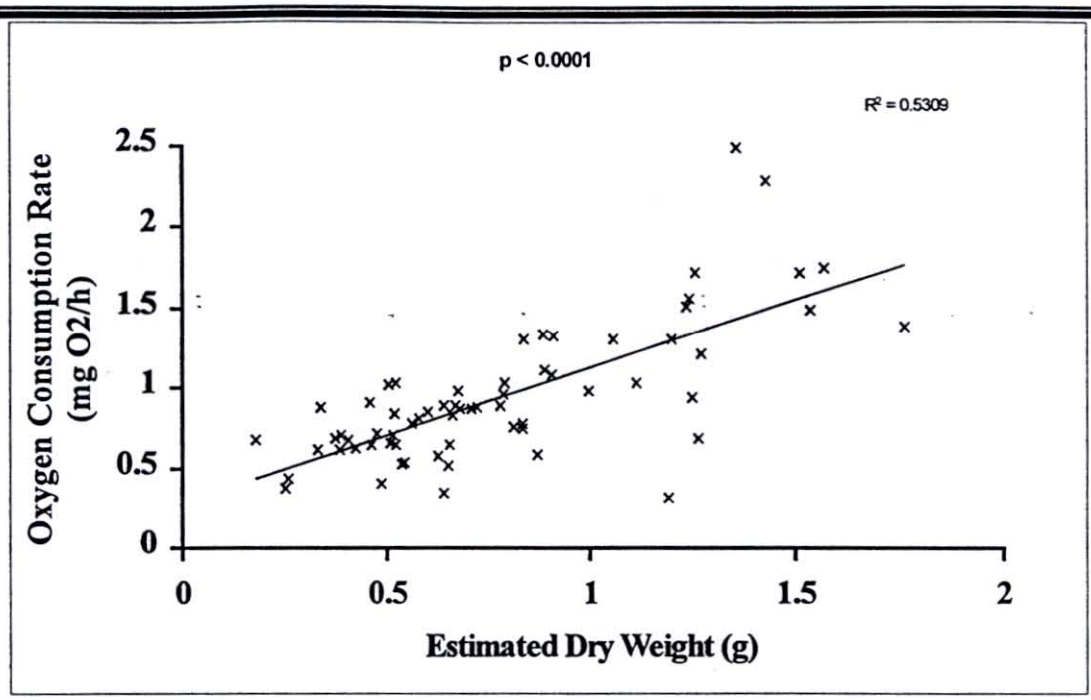


Fig. 4. Relationship between oxygen consumption rate and estimated dry weight.

Relationships between nutritive physiological parameters and dry weight, as shown for example for scope for growth (Fig. 5), were not, yet, easy to explain. Indeed, two responses of the animals to low food availability could be considered, one positive (SFG values increased) and the other negative (SFG values decreased) with increasing dry weight.

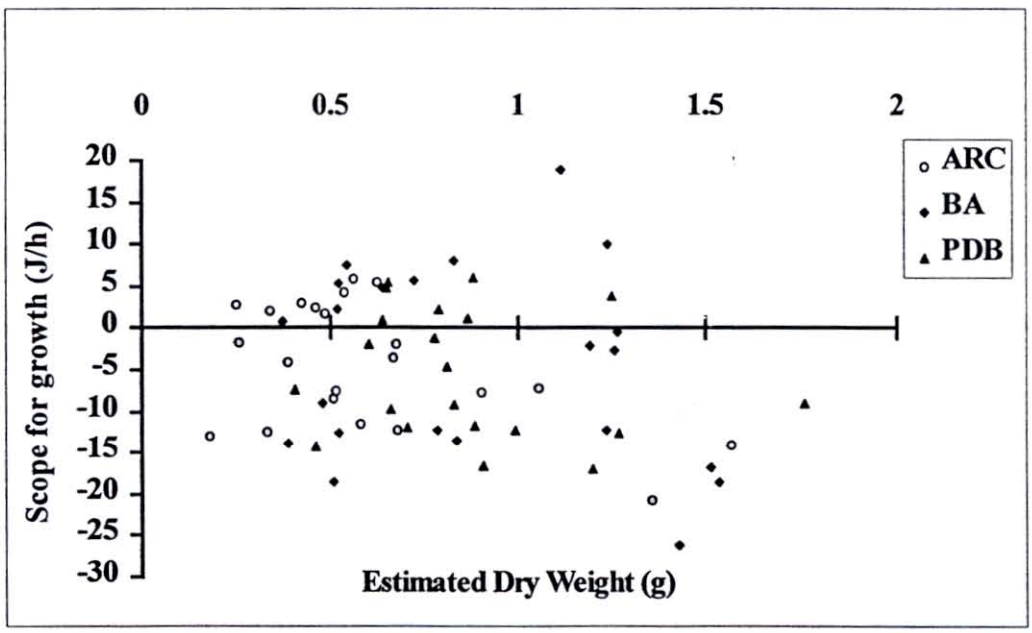


Figure 5: Individual scope for growth fitted to estimated dry tissue weight

4. Preliminary discussion and conclusion

The dry weight was not directly available in this experiments since the oysters have been dissected for genetical and physiological analyses. The relationship between total and dry weight is very strong as usually shown in laboratory reared populations. However, the population i.e. the type of intrapopulation cross affects significatively this relationship. This can be explained by the differences in the growth rate found between the populations, which has been shown to be a factor affecting the condition index in *Crassostrea virginica* and *Argopecten iradians*.

A linear relationship(OCR = aDW + b) between oxygen consumption (OCR) and dry weight (DW) was noticed. That means that oxygen consumption increased regularly with temperature. Such result seems to be in contraction with those of the literature. Indeed, authors, as Bougrier et al. (1995), showed a power relationship ($OCR = aDW^b$), meaning that the smaller animals consumed oxygen proportionally more than larger oysters. Such difference could be explain by the fact that, with about the same scale of dry weight, in this experiment, relationship was established from animals of the same age-class (estimated dry weights and low food availability), while Bougrier et al. (1995) established relationship from animals (observed dry weights and large food availability) of different age-class (from 1 to 3 years) oysters.

Those preliminary results suggested that the oyster response to low food availability could induced two different behaviour. Indeed, it seemed that the relationship between scope for growth and dry weight could be positive for some oysters, and negative for the others, even all these animals were reared in the same environment, from the fertilisation to the experimental date.

At present, no explanation was found. More data treatments have to be done in order to understand such behavioural differences.

5. References

Anonymous. (1987). Bilan énergétique chez les mollusques bivalves. Groupe de travail, La Tremblade. *Vie Mar.* **7**: 1-68.

Bardouil M, Bohec M, Bougrier S, Lassus P, and Truquet P. (1996). Feeding responses of *Crassostrea gigas* (Thunberg) to inclusion of different proportions of toxic dinoflagellates in their diet. *Oceanol. acta.***19**: (2) 177-82.

Bayne B.L. and Newell R.I.E. (1983) Physiological energetics of marine molluscs. In: *The mollusca Physiology Part 1*, edited by Saleuddin A.S.M. and Wilbur K.M. Plymouth, UK, NERC **4**: 407-515.

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 os stress and pollution on marine mussel. Praeger Scientific, New York, 384p.

Bougrier S. Geairon P. Deslous-Paoli J.M. Bacher C. Jonquières G. (1995) Allometric relationships and temperature effects on clearance and oxygen consumption rates in

Crassostrea gigas (Thunberg). *Aquaculture* **134**: 143-154.

Hawkins A.J.S., Smith R.F.M., Bayne B.L., Héral M. (1996). Novel observations underlying fast growth of suspension-feeding shellfish in turbid environments: *Mytilus edulis*. *Mar. Ecol. Prog. Ser.*, **131**, 179-190.

Rheault RB and Rice MA. (1996). Food-limited growth and condition index in the eastern oyster, *Crassostrea virginica* (Gmelin 1791), and the bay scallop, *Argopecten irradians irradians* (Lamarck 1819). *J. Shellfish Res.* **15**: 271-283.

SAS Institute Inc. (1988) *SAS/STAT User's Guide, Release 6.03*, SAS Institute Inc. 1-1028.

Urrutia M.B., Iglesias J.I.P., Navarro E. and Prou J. (1996). Feeding and absorption in *Cerastoderma edule* under environmental conditions in the bay of Marennes-Oleron (western France). *J.Mar.Biol.Ass.U.K.* **76**(2): 431-450.

Whyte J.N.C. (1987). Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. *Aquaculture* **60**(3-4): 231-241.

Sub-task 4.4 : Measurement of the digestive enzyme activity,

1. Introduction

This report deals with digestive enzyme measurements (amylase and laminarinase specific activities) on oysters from

- Generation G0 from four origins, Arcachon, Port des Barques, Seudre and Bonne Anse in France, sampled at two periods (April and June 96), to study differences between origins and seasons.

- Generation G1 from three 5X5 crosses intra populations of Arcachon, Port des Barques and Bonne Anse in France, to study differences between these three origins.

- Generation G1 to study relationships between absorption yield, ingestion, digestive enzymes and growth performances.

2. Materiel and Methods

Sample preparation : 20 oysters per origin (Arcachon, Bonne Anse, Port des Barques, Seudre) were sampled at random. Individual digestive glands were sampled and frozen in liquid nitrogen. Samples were ground in a Dangoumeau grinder with liquid nitrogen. The obtained powder was distributed and homogenised with distilled water for laminarinase analysis, with CaCl2 0.02M solution for amylase analysis and with NaOH 1N solution for total protein analysis (one night at 4°C).These samples were frozen at -20°C until analyse.

Digestive enzyme analyses were performed on the supernatant after centrifugation. Amylase and laminarinase activities were measured with an autoanalyser. Amylase was measured according to Samain et al (1985) protocol adapted to molluscs : the CaCl2 0.02M extract was added to a starch solution (1mg/ml) at pH 6.2 and 45°C. The starch decrease was measured by I2 and KI method. Laminarinase was measured according to a method developed in the laboratory : the extract is allowed to react on a laminarin solution at 2mg/ml, at pH 4.8 and 35°C. The increase of reducing sugars is measured with neocuproine method. Total proteins were measured by Lowry method, in NaOH 0.1N (1956)

For G1 samples, only 1/4 of the digestive gland was available, due to other studies for Genephys partners.

Digestive enzyme index : it was necessary to build an enzymatic index to simplify the expression of the two digestive enzymes. Because units cannot be compared, laminarinase is multiplied by a coefficient of 5 which gives a mean laminarinase value equivalent to the amylase mean. So the digestive index is defined as the sum (A/P +5*La/P).

Physiological measurements of ingestion (ING) and absorption rates (ABS)were performed at CREMA l'Houmeau (task 4.2). Values were divided by the dry weight of animals to standardise measurement to a standard oyster. They are expressed in

mg/h/g DW. Growth rates were measured at La Tremblade (Task 3). Growth was estimated during the last growing period just before experimentation in June. The slopes on the last three observations (J 325 to J385) were measured. Growth rate (GR) was expressed as the relative growth rate : increase in weight in 60 days (measured by the slope) on the initial weight at day 325.

3. Results

3.1. Analyses of digestive equipment in G0 populations:

According to the preceding report, a first set of 20 oysters from each of the four G0 populations reared in the same site till November 95, was analysed in April and June 1996. Individual data of amylase (A/P) and laminarinase (La/P) specific activity are in annexe 1. Results are in the table 1.

	La/P		A/P	
	April	June	April	June
Arcachon				
m	0.122	0.057	0.397	0.312
SD	0.059	0.015	0.129	0.088
Confidence interval (p=0.05)	0.055	0.027	0.170	0.140
Relative error	0.451	0.462	0.428	0.450
Bonne Anse				
m	0.085	0.049	0.361	0.280
SD	0.020	0.017	0.089	0.081
Confidence interval (p=0.05)	0.038	0.022	0.160	0.123
Relative error	0.450	0.450	0.443	0.438
Port des Barques				
m	0.126	0.054	0.318	0.344
SD	0.053	0.018	0.098	0.127
Confidence interval (p=0.05)	0.058	0.024	0.140	0.151
Relative error	0.462	0.450	0.440	0.438
Seudre				
m	0.095	0.045	0.485	0.282
SD	0.032	0.016	0.138	0.058
Confidence interval (p=0.05)	0.043	0.021	0.210	0.124
Relative error	0.450	0.462	0.433	0.438

Table 1 : Means, SD, Confidence interval, Relative error of specific amylase and laminarinase activities from 20 oysters per origin, and from 4 origins

Scattering of the values : analytical error has been established to be less than 10% with our methodology. The observed relative errors around the means in the samples were between 42% to 47%. They are large, but similar for all the samples from each origin, each period and each digestive activity measured (amylase and laminarinase).

Comparison of different origins : a variance analysis using an LSD test (less significant difference) is reported in tables 2.

Sites	April 1996			June 1996		
Enzymatic activity	A/P	La/P	A/P+5*L/P	A/P	La/P	A/P+5*L/P
Arcachon	b	b	ab	ab	b	b
Bonne Anse	ab	a	c	b	ab	ab
Port des Barques	a	b	a	a	b	b
Seudre	c	a	bc	b	a	a

*Table 2 : Difference between origins : A/P and La/P respectively specific activities of amylase and laminarinase, digestive index (A/P +5*La/P). Variance analysis, differences are significant at 95% confidence when letters are different (LSD test).*

Variance analysis (table2) demonstrated that two sites : Bonne Anse and Seudre had similar characteristics, and were sometimes different from the other sites (Bonne Anse in April was significantly different from Arcachon and Port des Barques, Seudre in June was different from the same two sites)

Comparison of seasons :

Amylase	**Arcachon**	**Bonne Anse**	**Port des Barques**	**Seudre**
April	a	a	a	a
June	b	b	a	b
Laminarinase				
April	a	a	a	a
June	b	b	b	b
A + 5*La				
April	a	a	a	a
June	b	b	b	b

*Table 3: Difference between seasons : A and La respectively specific activities of amylase and laminarinase or digestive index (A/P +5*La/P). Variance analysis, differences are significant at 95% confidence when letters are different (LSD test).*

The two digestive enzyme levels in April were significantly different from those observed in June in all the sites (Table 3).

Discussion :

Scattering : animals from the different origins were adapted till November 95 to the same environmental situation (same « claire »). The large scattering of data around the means reflects the large diversity of individual response of digestive enzyme levels to the same trophic situation. This diversity of response can be attributable to an individual specific ingestion behaviour, in time and/or quantity, or to a difference in capacity of individuals to synthesise these enzymes faced to the same food level. Oysters from this G0 generation are from a large mixture of animals of different age or environmental situation. Age differences can induce changes in ingestion behaviour as in digestive equipment, in relation to requirements of the animal. Environmental situation could also mark on a long term basis the behaviour and major metabolic pathways through an adaptation process. Possibly is this latter case, these adaptive differences can originate from the expression of some alleles genes. At last probably some of the scattering can originate from true genetic differences.

This large distribution offers possibilities to select among these individuals, oysters demonstrating a better digestive equipment faced to the same ingested food, or oyster demonstrating a long term adaptation capacity to induce allelic genes.

Seasonally : digestive enzyme specific activities vary according to seasons, in relation to trophic situations and feeding levels, as previously observed, during an annual cycle in Marennes-Oleron (Fig.1). These seasonal changes in trophic levels could explain the global changes in enzymatic levels whatever the sites and the enzyme activity considered.

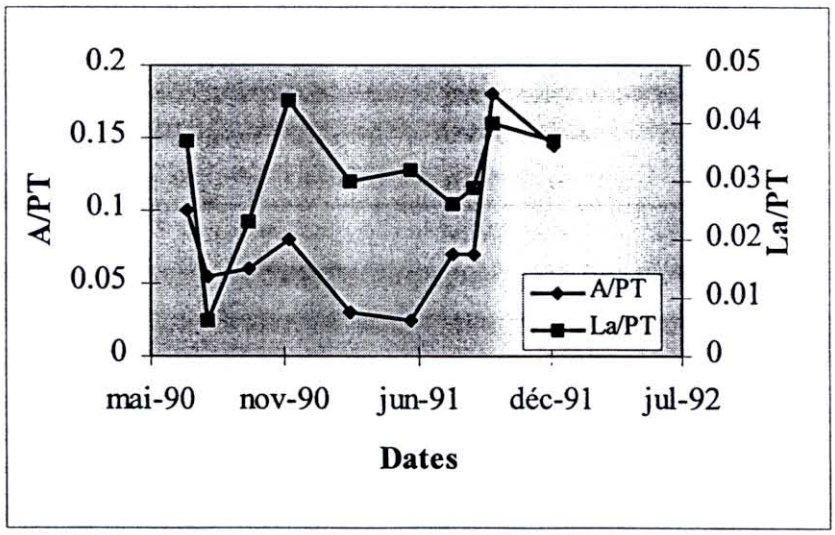


Fig.1 : Amylase and laminarinase specific activities during a year cycle in Marennes-Oleron Bay

Origin : the oysters from the sites of Seudre and Bonne Anse are in between the two other sites. They have some similarity. As they were adapted from November 95 to the

same trophic environment, they react in the same way to the seasonal effect.

Age effect: looking at the size structure of these populations it was also evidenced in the first report p29, that Seudre population and a part of the Bonne Anse population could be one year younger than Arcachon or Port des Barques ones. Age distribution similarity can explain that oysters from these two sites are similar in their digestive equipment but different from the oysters of the other sites because requirements levels vary with age, and can affect feeding behaviour and digestive regulations (Samain et al 1985, 1991, 1994)

3.2. Analyses of G1 populations :

The G1 generation from the 5X5 crosses in the three selected populations of Port des Barques, Arcachon and Bonne Anse was reared in La Tremblade in non limiting food, and without trophic competition. Before non destructive measurements of physiological parameters (see CREMA), they were adapted 15 days to a more limiting condition (2 mg/l) and a lower temperature (15°C).

We have measured digestive enzymes on 1/4 of the digestive gland. Means and statistical data are reported in table 4.

		La/PT UI/mg	A/Pt mg/mg
PDB	Mean	0.119	0.584
	SD	0.026	0.162
	Confidence interval (p=0.05)	0.011	0.073
	Relative error	0.10	0.12
ARC	Mean	0.109	0.653
	SD	0.033	0.200
	Confidence interval (p=0.05)	0.014	0.083
	Relative error	0.130	0.13
BON	Mean	0.118	0.750
	SD	0.029	0.211
	Confidence interval (p=0.05)	0.013	0.092
	Relative error	0.11	0.12

Table 3 : Mean, SD, Confidence interval and Relative error for specific activities of laminarinase and amylase, of 20 oysters from 5X5 crosses in three origins : Port des Barques (PDB), Arcachon (ARC), Bonne Anse (BON).

Scattering : each sample of 20 individual oysters from each G1 intrapopulation demonstrated a lower scattering compared to the original G0 ones.

Comparison of the means : no significant difference was observed on the mean

values of amylase or laminarinase specific activities in the G1 generation whatever the origin (Table 4).

	A	La	A +5*La
Port des Barques	**a**	**a**	**a**
Arcachon	**ab**	**a**	**a**
Bonne Anse	**b**	**a**	**a**

Table 4

Discussion :

Animals were coming from a cross of 5X5 animals of each origin, moreover they were reared in exactly the same conditions and have exactly the same age. As a consequence, a large decrease is observed in standard deviation which could be in relation to this specific common history and possibly to a lower genetic diversity than the original population, due to the limited number of crosses, which should be checked by genetic techniques.

In these well controlled trophic conditions, the lack of difference between the three origins would suggest that the three populations are very similar for the digestive enzyme character.

As a consequence, genetic differences should be studied at the level of individuals inside the populations.

3.3. Relationships between absorption yield, ingestion, digestive enzymes, and growth :

One hypothesis about growth variability is that absorption yield can vary greatly among individuals exhibiting the same ingestion rate.

This is illustrated by the 97 results when all the 60 samples from the three origins were analysed (Fig.2):

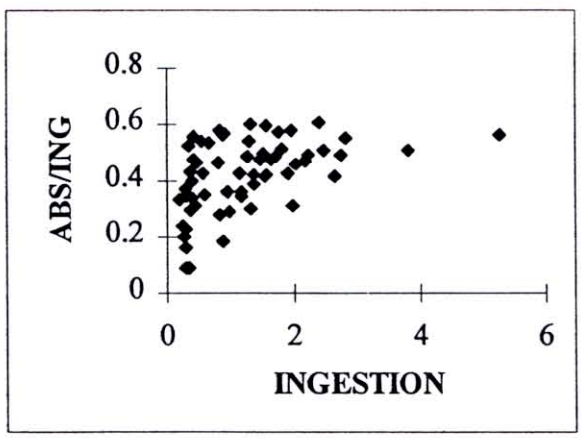


Fig.2 : Absorption yield = Absorption rate/Ingestion rate (ABS/ING), versus ingestion rate (in mg/h/mg DW). 60 oysters from the three G1 subpopulations.

These results show that absorption yield varied with ingestion rate in an asymptotic way, with a maximum value around 60%, and a large variability between 0 to 3 mg/h/gDW. Individuals with the same ingestion rate demonstrated a large variability in their absorption yield. we suggest that digestive equipment can explain these absorption yield differences.

The results observed in Fig (2), looks like a mixture of Michaelis-Menten curves with different values for (E) and different values for Km. The hypothesis that digestive enzyme levels can explain differences in assimilation yield can be tested by dividing ABS/ING by (E).

We obtain two sets of Michaelis curves, which can be transformed according to a Lineweaver and Burk transformation :

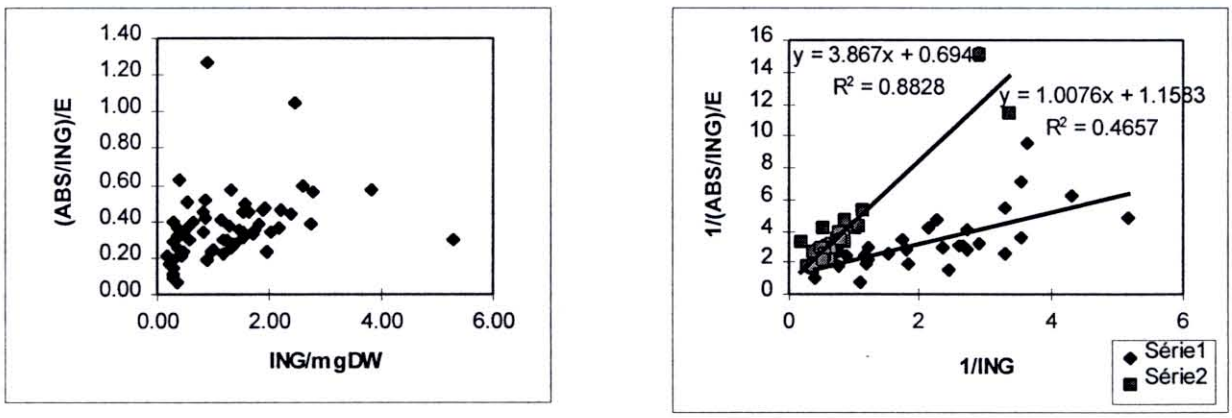


Figure 3

This transformation gives two lines corroborating the existence of two sets of data.. These two sets demonstrated a similar Abs Max, but different Km. According to these results a possible model could be suggested :

$$\mathbf{ABS/ING = (ABS/INGmax)(E)(ING) /(Km + ING) \quad (1)}$$

From these first results, animals from each set were identified. The data appeared well distributed in two parts of the graph. The dispersion observed in each set can be attributable to changes in enzyme levels inside the set, or to other intermediate sets with different Km.

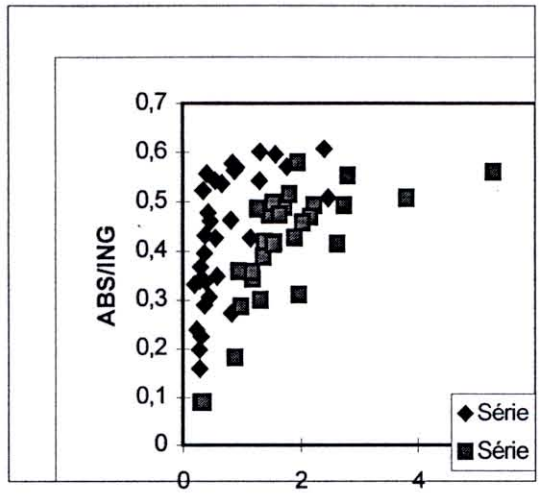


Figure 4

According to the proposed model, we simulated for each set of data the absorption yield using the two different « Km like » values in the equation (1).Two sets of absorption yield were obtained. As only one predicted value should be selected per sample, the smallest difference between the predicted value and the observed value was used to classify data in one Km class.

Vm value can be estimated from observed data : maximum observed absorption yield = 0.60. This value was observed for a mean in digestive enzyme activity of 1.24. So ABS/ING max value for 1 enzyme unit is .60/1.24 = 0.44. A value of 0.50 gave a good fitting to data. Simulations are reported in Fig.(5).:

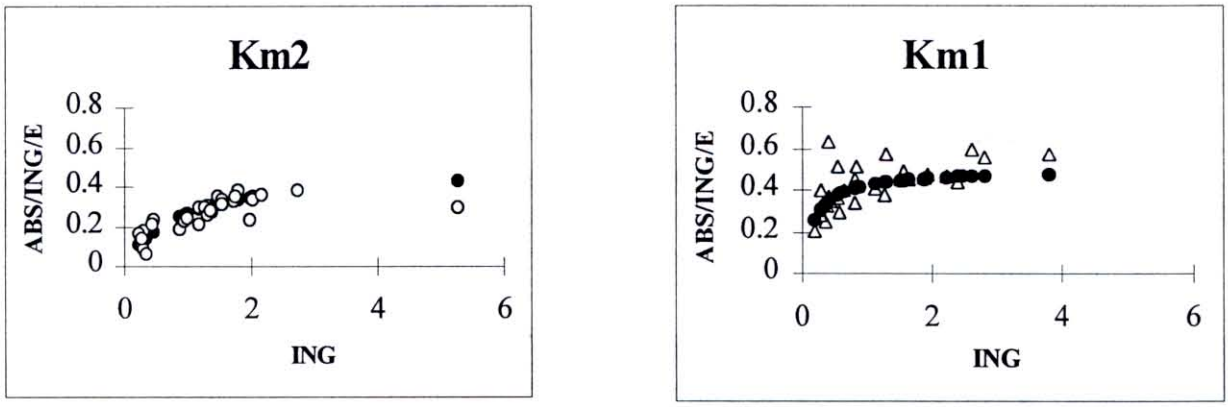


Fig.5 : Absorption yield per enzyme unit, versus ingestion rate. Dots = simulation, empty circles = observed data. Km1 = .18, Km2 = .86, ABS/ING max = .50

Correlation between predicted and observed ABS/ING data is significant at p = 0.005. However, some high values were not well predicted, suggesting that a better adjustment should be studied.

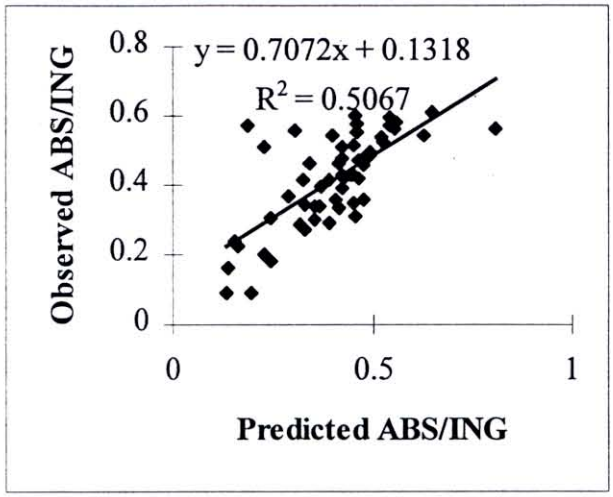
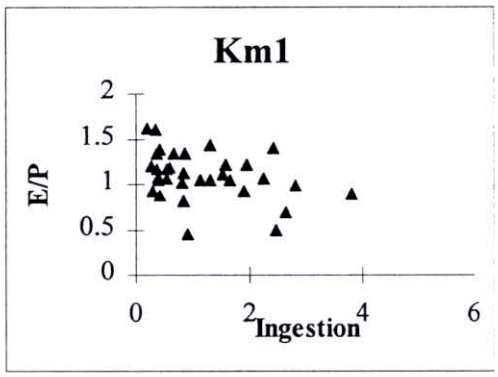


Fig.6 : Correlation between observed and predicted absorption yield

This first approach allow us to study the properties of these two groups of data :



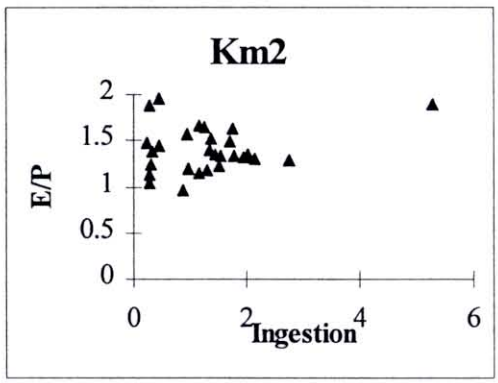


Fig.7 : Specific digestive index versus ingestion rate (mg/h/mg DW) in each set of data characterised by a Km1 or Km2 value (respectively 0.18 and 0.86mg/h/mg DW)

The means of the digestive equipment of the two groups (E/P Km1 group = 1.40, E/P Km2 group = 1.10) are very significantly different (T test p inf.0.001). Distribution of data showed different trends with ingestion rate. In group Km1, E/P trends to decrease at high ingestion values, in group Km2, E/P trends to increase at high ingestion values.

The relationships between growth rate and ingestion rate are also different :

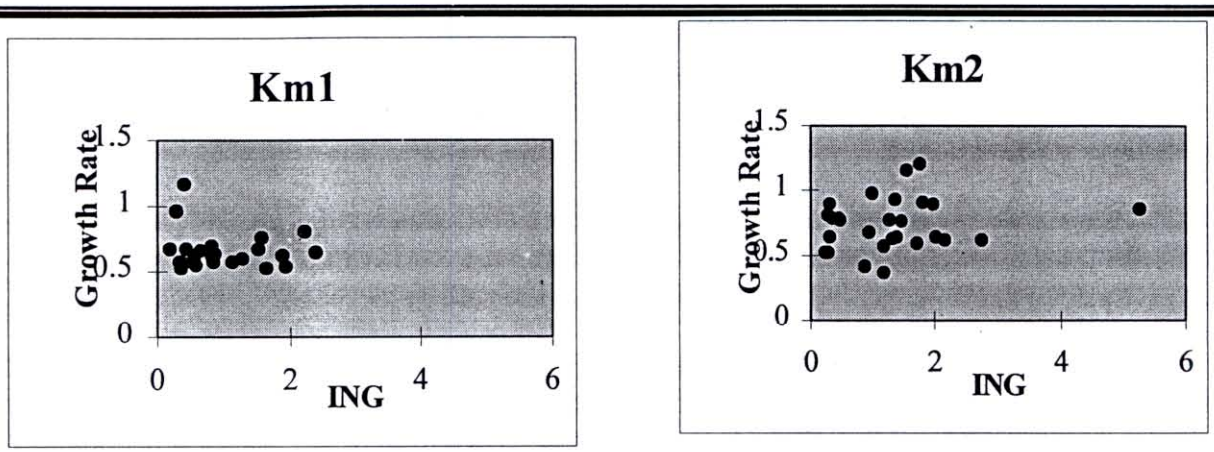


Fig.8 : Growth rate versus ingestion rate (mg/h/mg DW) in each set of data characterised by a Km1 or Km2 value (respectively 0.18 and 0.86mg/h/mg DW)

Growth rate of group Km1 was low, except for two samples. Group Km2 demonstrated more variable growth rates between 1 and 2 mg/h/gDW of ingestion rate than group Km1. The means (respectively 0.69 and 0.74) were not significantly different (T test p = 0.37).

Simulation demonstrated that ABS/ING in group Km1 was higher than in group Km2 at low values of ingestion rate (less than 1mg/h/gDW), identical between 1 and 2 mg/h/gDW, but decreased with ingestion rate thereafter and were lower than in group Km2 over 2 mg/h.

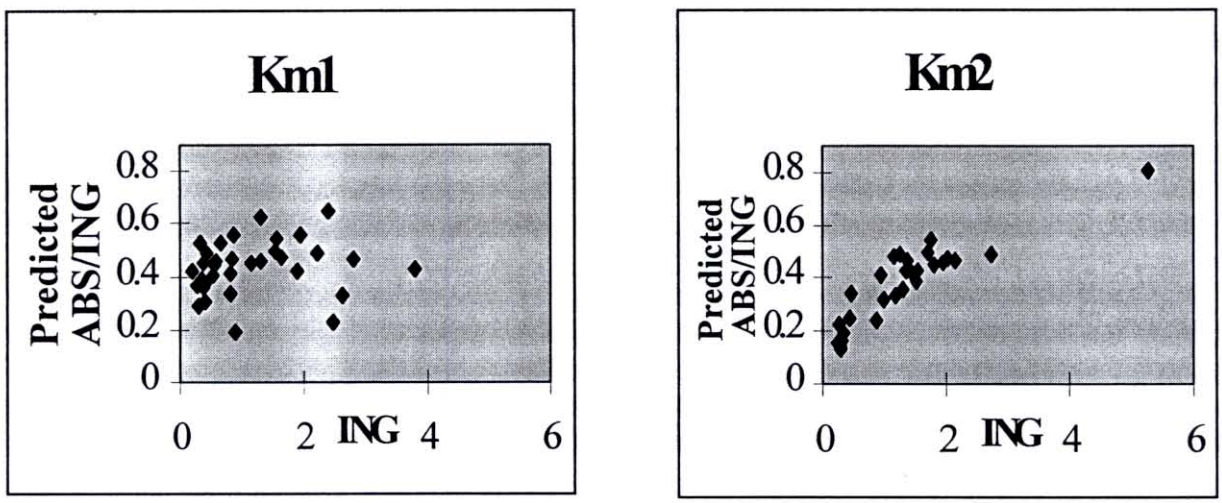


Fig.9 : Predicted absorption yield ABS/ING versus ingestion rate (mg/h/mg DW) in each set of data characterised by a Km1 or Km2 value (respectively 0.18 and 0.86mg/h/mg DW)

Discussion :

Relationships between absorption yield and ingestion rate showed that different samples from oyster demonstrated different absorption yield with a same ingestion rate. Such a result has been reported by Bayne (1983) for growth rate and ingestion rate in the mussel *Mytilus edulis*. Different explanations were suggested. Some authors tested

the effect of organic/inorganic ratio in the food on absorption yield (Deslous-Paoli et al. 1992). In GENEPHYS experiment, oysters were fed exactly the same food, so the organic/inorganic ratio was the same for all of the oysters .We suggested that genetic differences in digestive enzyme equipment could be at the origin of these observations.

Our preceding work on Artemia and on scallop larvae led us to the conclusion that the three variables , absorption, ingestion and digestive enzymes were linked together in a Michaelis-Menten like expression (Samain et al. 1985,1991):

Data from G1 oyster generation were analysed using the same model. Relationships between absorption and ingestion, demonstrated that absorption yield was better related to ingestion and digestive enzyme activity, than absorption alone. So absorption yield was substituted to absorption in the original equation which turns in :

ABS/ING = (ABS/INGmax)(E)(ING) /(Km + ING) (1)

Where ABS/ING = Absorption yield, ING = Ingestion rate, E= Digestive enzyme specific activity (index A/P+5*La/P), ABS/INGmax = maximum absorption yield constant, Km = Affinity constant like

The Lineweaver-Burk transformation gave two linear regression lines. Two different Km like constants were obtained suggesting two digestive systems with different affinities. The ABS/INGmax constant observed (equivalent to the dissociation constant k2 in enzymology) was of the same order for the two sets of data.

The data could be explained by the existence of two different sets of animals. One whose absorption efficiency increased very fast with the ingested food. This high efficiency is marked by a small Km like value (Km1 = 0.18 in set1) of a digestive enzyme system. In the second set of data, absorption efficiency increased more slowly, corresponding to a digestive enzyme system with a lower apparent affinity (Km2 = 0.86 in set2). Moreover, in each digestive system, enzyme levels can vary. The two phenomena could explain the data distribution observed in Fig (2).

Using these two Km like characteristics, and one value of ABS/INGmax constant, it was possible to modelise absorption yield with ingestion rate and digestive enzyme levels. A very significant correlation between observed and predicted data was obtained corroborating the model. To our knowledge, it is the first time that absorption efficiency has been modelised using ingestion rate and digestive enzyme parameters.

The origin of these differences in affinity should be studied. We suggest to test the hypothesis that these two groups of digestive systems are relevant to two groups of digestive enzyme genes with different kinetic parameters. Numerous genes of amylase were described in Drosophyla (Da Lage et al.1992, Inomata et al.1995) or in shrimps, some amylase genes seem to be expressed in relation to substrate concentration or to development stage (Van Wormhoudt et al.1996). We have recently evidenced at least two amylase genes in C.gigas (URM14), with differences between individuals. Differences in digestive enzyme gene equipment is opened for study. To verify our hypothesis, a molecular approach is planed to characterise amylase genes in the same 60 samples, if available. Km determinations will be performed on the corresponding

samples.

Properties of the two groups of samples : group Km1 had lower, digestive activities than group Km2. A possible explanation is that digestive enzyme synthesis is repressed when ingestion rates are over the Km value (Samain et al 1985,1991, Inomata et al 1995). This repression is not evidenced in group Km2 data because Km2 is 4 time higher than Km1. In this last case, only an activation process is observed.

Growth rate and Km groups :

The similarity of the means for the growth rates of the two groups seems surprising (Fig.8). We should expect that the group with a low Km value, which had a maximum absorption efficiency at small ingestion rates, would grow faster than the group with high Km value. In fact, a low digestive equipment is associated to the low Km group and absorption yield is only maximum when ingestion is low, but decreased thereafter. On the contrary, absorption yield of group Km2 is high when ingested food is high. According to these observations, animals from group Km1 would grow faster than animals from group Km2, in low trophic conditions. On the contrary, in higher trophic conditions, group Km2 would grow higher than group Km1.

4. General conclusion

Digestive enzymes of G0 generation varied normally according to origins and seasons. A large standard deviation of 45% was observed on the data from each site at one sample date for both enzymatic activities (amylase and laminarinase). This deviation is reduced to 12% for the G1 generation. This large reduction is attributable to a higher homogeneity in environmental conditions for the G1 generation, especially with the limitation of trophic competition. But this cannot exclude a possible reduction in genetic heterogeneity, as only 5X5 crosses were performed in each site.

Means of digestive activities in G1 oysters from the three parental origins were not significantly different. Standard deviations were of the same order in each oyster subpopulation. As a consequence, one of these subpopulations cannot be selected on the basis of digestive activities, to obtain specific physiological performances. But the selection should be performed on individual performances.

Individual absorption yield greatly depends on ingestion rate, with a large variability at low ingestion rates. This result showed that individuals, faced to the same food availability, demonstrated 1/ different ingestion behaviour, and 2/ different absorption yield, when ingestion rate was the same. These criteria are two possible sources of selection.

A Michaelis -Menten model is proposed to modelise absorption yield as a function of digestive enzyme levels and ingestion rate. Two sets of data can be separated, depending on two different digestive systems, one is characterised by a high affinity to ingested food (group Km1 low), the second by a low affinity (group Km high). Using these kinetic parameters in the model, predicted absorption yields were highly correlated to observed data ($p=0.005$) Samples from the first set demonstrated lower enzymatic activities than those from the second set, as well as a different growth rate

distribution with ingestion rate compared to the second one.

This model predicts that oyster with a high affinity digestive system are well adapted to low trophic situations, compared to other oysters with low affinity, but their growth rate will be slow in relation to low ingestion rates and low digestive enzyme activities. On the contrary, oysters demonstrating a low affinity digestive system are well adapted to high trophic situations. Their growth very low at low food ingestion rate, is higher when ingested food is high, because simultaneously, their absorption efficiency becomes high, as well as the levels of digestive enzymes

Significance of high and low efficiency digestive systems will be studied 1/ at the level of catalytic properties of amylase and laminarinase of selected samples and 2/ if possible at the genetic level, as amylase is coded at least by two different genes in *C.gigas* oysters, differently represented in individuals.

This model will be used to study in more detail results of G1 generation from the three origins.

5. References :

Bayne B.L., 1983 : Physiological ecology of marine molluscan larvae. IV Growth. In: *The mollusca*, Academic Press, **vol 3**, 299-343.352p.

Da lage J.L., Lemeunier F., Cariou M.L. and David J.R. 1992 : Multiple amylase genes in Drosophila ananassae and related species. *Genet.res.Camb.,* **59**, pp 85-92.

Inomata N., Kanda K., Carion M.L., Tachida H., Yamazaki T. 1995 : Evolution of the response patterns to dietary carbohydrates and the developmental differenciation of gene expression of alfa amylase in drosophila. *J.Mol.Evol.,* **41**, pp 1076-1084.

Deslous-Paoli,J.M., Lannou A.M., Geairon P, Bougrier S. Raillard O., Héral M. 1992 : Effects of feeding behavior of Crassostrea gigas (bivalvia mollusca) on biosedimentation of natural particulate matter . *Hydrobiologia*, **231**, pp 85-91.

Samain J.F., Hernandorena A., Moal J., Daniel J.Y. and JR. Le Coz, 1985 . Amylase and trypsin activities during Artemia development on artificial axenic media : effect of starvation and specific deletions. *J.Exp.Mar.Biol.Ecol.*, **86**, pp 255-270.

Samain J.F., Salaun M., Diouris M., Daniel J.Y., Le Coz J.R., Moal J., 1991. Digestive enzymes during development of Pecten maximus, relations with ingestion and absorption processes. 13th. International Congress of E.S.C.P.B. : Research for aquaculture, fundamentals and applied aspects. Antibes-Juan Les Pins. 6-10 Octobre 1991.

Samain J.F. (1994). Enzymes digestives et croissance . Quelles perspectives? 3ème colloque de biotechnologies marines. Concarneau.

A.Van Wormhoudt, Le Moullac G, Klein B., Sellos D. 1996. Adjustement of the expression of digestive enzyme to casein level in food in *Penaeus vannamei*, Brit.J.Nutr. (Proc.Nut.Soc.), *Cambridge press*, 55, abs. **14A**

6. Annexe 1 :

KM2 = LOW AFFINITY AT LOW FOOD INGESTION							Population Km2	
N° tube	Populatio	IND	I (mg/h/g)	Abs (mg/h/g)	A/P+5*La/P	croissance relative su	PrediABS/ING	ObsABS/ING
9	PDB	05c9	0,877761739	0,160428273	0,9655457	0,417336034	0,243853646	0,182769726
56	PDB	05f6	1,71651949	0,837059719	1,48797814	0,594924541	0,495657706	0,487649411
43	ARC	09e5	2,737077902	1,347633906	1,29349967	0,617898514	0,492122976	0,492362276
41	ARC	09g1	1,179265257	0,40411492	1,14975295	0,368099321	0,332439269	0,342683648
44	ARC	09g6	5,275498019	2,96878659	1,88433166	0,853767322	0,810104405	0,56
19	BON	13f8	2,026896927	0,926955142	1,35478177	0,638053833	0,475597688	0,457327223
28	PDB	06c6	2,163012053	1,01530262	1,29946333	0,623853211	0,464893092	0,469392955
55	PDB	05d4	0,3031161	0,0683524	1,23842635	0,645112192	0,161371236	0,225499075
14	PDB	05g1	0,231782541	0,055501455	1,48021804	0,51873269	0,157123184	0,239454856
10	PDB	06b8	0,275097386	0,05471975	1,88183313	0,527267378	0,228036546	0,198910468
26	ARC	08f6	1,375556055	0,576565967	1,51327599	0,648760699	0,465565591	0,419151197
22	BON	13e3	1,167517188	0,417576046	1,66185567	0,57067323	0,478478079	0,357661583
50	BON	14g2	1,263102137	0,613642664	1,63745094	0,772979956	0,487086266	0,485821887
32	PDB	05b6	1,460260024	0,692288802	1,34334371	0,758874992	0,422717949	0,474085978
47	ARC	08f8	1,312601706	0,39403997	1,17087444	0,626616356	0,353698466	0,300197667
8	ARC	17h1	1,532384752	0,633764064	1,21718431		0,389819129	0,413580247
67	BON	15a6	1,966681778	0,611317834	1,31223922	0,892719743	0,456499381	0,310837188
63	BON	15b3	1,804463434	0,929909701	1,3344	0,899157092	0,451850075	0,515338623
15	BON	15g8	0,946496929	0,33903723	1,55932487	0,684056061	0,408496736	0,358202145
39	BON	13e9	0,465635978	0,214518421	1,95659467	0,77488271	0,343631619	0,460699841
42	ARC	08g3	0,44343846	0,136005617	1,44758583	0,790214673	0,246239178	0,30670686
46	ARC	07h5	1,551083598	0,648227925	1,3372859	1,150232696	0,430147306	0,417919399
16	ARC	07f6	0,297605176	0,026926214	1,03716216	0,896216102	0,133320425	0,090476296
36	ARC	08c6	1,359293867	0,528462772	1,38889945	0,933771937	0,425343063	0,388777427
49	ARC	08d1	0,985318029	0,282319601	1,19300471	0,977650642	0,318505817	0,286526373
51	ARC	09a3	1,758473047	1,005228528	1,62081851	1,200105352	0,544241931	0,571648528
20	BON	13g8	0,342563876	0,03118151	1,38041734	0,785618115	0,19661372	0,09102393
31	ARC	08c8	0,282447071	0,045067146	1,13043478	0,81	0,139738637	0,159559615

KM1 = HIGH AFFINITY AT LOW FOOD INGESTION							Population Km1	
N° tube	Populatio	IND	I (mg/h/g)	Abs (mg/h/g)	A/P+5*La/P	croissance relative su	PrediABS/ING	ObsABS/ING
57	PDB	06h3	1,638893228	0,776523074	1,0489547	0,533390027	0,472574402	0,473809435
5	ARC	07g1	1,943620591	1,125877733	1,21782497	0,542941296	0,557300512	0,579268268
64	BON	15d5	1,893215078	0,808953193	0,92602225	0,619774997	0,422811725	0,427290698
25	PDB	05a3	0,383386388	0,129497422	1,04796696	0,559358539	0,356572574	0,337772613
27	PDB	06a6	0,577951729	0,201490915	1,19158692	0,550622879	0,454303154	0,348629314
7	ARC	09b8	0,550604109	0,23493303	1,17677029	0,60022427	0,44342384	0,426682304
48	ARC	16a8	0,375408094	0,162088131	1,33327759		0,45059048	0,431765146
18	BON	13b4	0,655483871	0,351520218	1,33482623	0,665320117	0,523622952	0,536275922
60	BON	15a2	0,344014106	0,179668225	1,6	0,572489566	0,525198237	0,522269937
24	PDB	05a8	0,811329871	0,375466331	1,01446417	0,700748755	0,415131788	0,462778882
53	PDB	05g2	1,293735765	0,701685898	1,4288786	0,59552022	0,627178694	0,542371879
54	PDB	06h9	0,368208383	0,106768094	1,16246805	0,53211282	0,390390311	0,289966495
6	ARC	09c2	0,840646341	0,486024078	1,12049593	0,571054573	0,461443287	0,57815523
29	ARC	09e6	2,400954021	1,458647766	1,39275198	0,647831642	0,647809578	0,607528405
61	BON	14b2	0,870756096	0,490524816	1,34206335	0,641511053	0,55608045	0,56333205
34	BON	14g6	1,567858775	0,934987769	1,21243898	0,758755487	0,543789098	0,596346931
23	PDB	06h4	0,824265331	0,225165174	0,81009201	0,627072871	0,33244738	0,273170745
12	PDB	06h6	1,141886816	0,487252503	1,03902116	0,574379873	0,448769348	0,42670823
45	PDB	05f9	2,227688796	1,097237764	1,05641322	0,802390482	0,488717622	0,492545353
30	PDB	05h2	1,519819976	0,756196137	1,10480349	0,674513583	0,493905956	0,497556387
13	PDB	17g2	0,301257713	0,110675215	0,92201835		0,288582532	0,367377198
40	BON	15h6	0,425914841	0,203258062	1,38159509	0,671163044	0,485581318	0,477227
37	BON	14f1	0,19328887	0,064081786	1,61552478	0,670644156	0,418259135	0,331533761
35	BON	15c6	0,282118585	0,095955239	1,20413349	0,96155638	0,36755548	0,340123778
17	BON	15g5	0,4	0,145238442	1,0798001	1,164849594	0,372344861	0,395436131
68	BON	15b9	0,407282496	0,22664766	0,87955548	0,525813222	0,304987425	0,556487604
59	BON	14f4	1,308076356	0,786683202	1,0419859	0,644757845	0,457972844	0,601404649
1	ARC	08e2	0,906273012	0,516752167	0,45045218	0,690074994	0,187905183	0,57
3	ARC	09c4	2,46857774	1,253276687	0,48690654	0,767242466	0,22690794	0,507691804
21	PDB	04h8	3,803990701	1,932658911	0,8870196	0,850838482	0,423471661	0,508060893
2	ARC	09f6	2,623703772	1,087373466	0,69549987	1,036612469	0,325424112	0,414442163
65	BON	14b4	2,800279511	1,547281477	0,98382625	0,971980923	0,462203037	0,552545369
33	ARC	07b7	0,547016429	0,296913627	1,06535726	0,764866699	0,400794193	0,542787404

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°2

NERC
Natural Environment Research Council

Plymouth Marine Laboratory

Reporting Period:
1st January-31st December 1997

ANNEX III
Individual Progress Report
Title page to participant n°2

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°2

Total cost to partner n°2 : 274.000 ECU

EC contribution : 137.000 ECU ***% of the total cost :*** 50

Duration : 60 months	***Commencement date :*** 1/1/1996 ***Completion date :*** 31/12/2000

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Coordination genetic aspect :
Dr Pierre BOUDRY
(IFREMER La Tremblade)

Coordination physiological aspect :
Dr Serge BOUGRIER
(IFREMER L'Houmeau)

Participant n°2

Natural Environment Research Council, Contractor Dr. A.J.S. HAWKINS

Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
United Kingdom

FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1997

Participant n°2 **Natural Environment Research Council (NERC)**

Plymouth Marine Laboratory
Prospect Place
Plymouth PL1 3DH
United Kingdom

Scientific team **Dr A.J.S. HAWKINS**
A.J. DAY
R.F.M. SMITH

Objectives :

- Measurement of physiological functions
- Data exploitation and synthesis.

Actions in the project :

Task 4	Physiological analysis
Sub-task 4.3	Measurement of the proteolytic activity and protein turn-over Realisation of the whole task by the Partner..
Task 8	Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis.
Task 9	General organisation of the project Participation in annual scheduling meetings.

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
4.3		Physiological analysis/ PR	Physiological analysis/ IS	Physiological analysis/ PR	Physiological analysis/ FR
8		Data processing/ PR	Data processing/ IS	Data processing/ PR	Final synthesis/ FR
9	2 meetings with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners

PR = Periodical report
IS = Intermediary Synthesis
FR = Final Report

Research activities during the second reporting period :

Task 4 : Physiological analysis

Sub-task 4.3 : Measurement of the proteolytic activity and protein turn-over,

1 INTRODUCTION

In the first year of our programme we optimised assays to compare differential expression of three stress-70 kDa and one stress-60 kDa stress protein isoforms. We also developed assays for leucine aminopeptidase, Cathepsin B, and Cathepsin D; three enzymes that are known to play an important role in protein turnover. In the case of Cathepsin D, we used a general proteinase substrate, haemoglobin, and isolated Cathepsin D activity by the addition of control incubations that included a specific inhibitor of aspartic proteases, Pepstatin A. We found, using this substrate, that activity was maximal over a wide range of pH from 2.2 to 3.8. In addition, although we could account for virtually all of the haemoglobinase activity present in the digestive gland (by the use of specific inhibitors), up to 40% of the activity in remaining tissues could not be identified. The occurrence of artifacts in the use of haemoglobin as a substrate is well known (Smith & Van Frank 1975), although these problems are generally encountered in assays that detect peptide products by UV (e.g. Peek 1987).

During this past second year of our programme, to rule out the possibility that our findings for Cathepsin D were due to artifacts associated with our choice of substrate, we have developed an assay that uses a synthetic peptide substrate, and compared results with those obtained for haemoglobin. We have also undertaken our first collaborative experiments whilst working with IFREMER scientists in France to compare protein metabolism within fast- and slow-growing oysters from each of the three G1 populations, in which growth and physiological traits have been monitored at different life-stages.

2 METHODS

2.1 OPTIMISATION OF ASSAYS FOR PROTEOLYTIC ENZYMES

Ten large (3-6 cm) oysters from Seasalter Ltd., Whitstable, Kent, were dissected into adductor muscle, gill, digestive gland and remainder. Each tissue was frozen in a single pool of 10 individuals. Tissues were homogenised with an Ultra-Turrax homogeniser in CMFS buffer pH 7.3 (1g tissue : 5ml buffer), and centrifuged at 15,000 g for 15 mins. Supernatants for each tissue were frozen in 1.5 ml aliquots and stored at -70°C.

Our first assay of Cathepsin D that used haemoglobin as a substrate (Barrett &

Heath 1977) has been described in our previous report. Our second assay uses a synthetic peptide substrate designed specifically to measure Cathepsin D activity. This synthetic substrate is Bz-Arg-Gly-Phe-Phe-Leu-4MβNA salt, henceforth referred to as Bz peptide. The assay using Bz peptide is carried out in two phases following the protocol of Smith and Van Frank (1975). In the first phase, Cathepsin D hydrolyses the substrate which is in 10 mM formate buffer adjusted to between pH 3.3 and 4.5. This reaction is stopped with 1% potassium hydroxide followed by the addition of 0.1M Hepes buffer pH 7.0, both of which elevate the pH. In the second phase, aminopeptidase M liberates 4MβNA from the peptide products. This second reaction is stopped after 1 hour with 1M HCl plus 2% Triton X-100 and post-coupled with Fast Blue B to produce a coloured product. The substrate forms a precipitate during the above reaction. Smith and Van Frank (1975) stated that this precipitate should not affect the enzyme-substrate reaction, and reported that the precipitate was solubilised by addition of triton X-100 to the stopping reagent. However, we found that the suspension remained, irrespective of the concentration of triton X-100. After the addition of Fast Blue B, we therefore centrifuged at 6,000 g to remove turbidity that might otherwise have interfered with spectrophotometric measurement. For both assays, activities were measured with and without pepstatin.

2.2 MEASUREMENT OF WHOLE-BODY PROTEIN TURNOVER IN FAST- AND SLOW-GROWING OYSTERS FROM EACH G1 POPULATION

2.2.1 Whole-body protein turnover

Whole-body protein synthesis was determined in 40 oysters, that represented among the faster- and slower-growers from each of the three G1 populations (Arcachon, Port des Barques and Bonne Anse). These 40 oysters were within a larger group of 70 that had been used for physiological measures in previous weeks. During this time, and for more than one month before our measures of protein synthesis, all oysters had been acclimated in a system of recirculating seawater of full salinity at 14.5 ± 1.0 oC whilst fed continuously on Skeletonema costatum that was available at about 2 mg l-1.

Rates of protein synthesis were determined in vivo by quantitatively administering each oyster 15N-labelled Skeletonema costatum for 3 h, and then monitoring the subsequent excretion of 15N as ammonium over 17 h according to an end-product analysis as described by Hawkins et al. (1987). Two measures were undertaken, each in consecutive weeks upon separate groups of 20 oysters, which were divided so that each group was representative of different populations and growth rates.

Protein turnover will be calculated as whole-body protein synthesis minus net protein balance computed on the basis of measured lifetime growth.

2.2.2 Dissection

(a) Oysters used for determinations of protein turnover and physiological traits (oyster stock B1.2)

In June 1997, seventy oysters were dissected, which included the forty oysters which had been used for determinations of protein turnover and physiological traits. These oysters were dissected into four tissue groups: digestive gland, adductor muscle, gill, and remaining tissues. All tissue groups were wet-weighed, whole, prior to further sub-division as in Table 1. All samples were frozen in liquid nitrogen and stored at -70°C. Unfortunately, samples of adductor muscle, digestive gland and gill destined for analysis of proteolytic enzymes and stress proteins (marked with **) were lost in transit from IFREMER to PML due to a faulty cool-box that allowed dry ice to be lost and samples to thaw. Remaining tissues were only partially thawed (marked with *), and have been retained. A control experiment will assess the effects of partial thawing and subsequent refreezing on enzyme activity in a different set of oyster tissues before analysing these samples.

Table 1. Dissection of tissue samples from June 1997

Sub-task		Proportion of tissue used for each sub-task			
		Digestive gland	Adductor muscle	Gill	Remaining tissues
Proteolytic enzymes	4.3	3/8**	1/2**	0	All*
Stress proteins	4.3	0	0	1/2**	-
Digestive enzymes	4.4	1/2	0	0	-
Allozymes	5.1	1/8	1/2	0	-
Microsatellites	5.2	0	0	1/2	-

(b) Oysters which were monitored for growth rate at Bouin (oyster stock R1)

The loss of the samples described above meant that we would have had no measures of stress proteins or whole-body activities of proteolytic enzymes that we could relate to growth. Therefore, in July 1997, sixty further oysters were dissected from G1 that had been monitored for growth at Bouin. Thirty of the fastest-growing (large) and thirty of the slowest-growing (small) oysters were selected and posted overnight to IFREMER-Ronce. On arrival, they were put into 2 insulated containers on ice-packs, and large and small oysters were dissected alternately. It has often been observed in shellfish that handling stress may trigger spawning in animals with ripe gametes. Therefore, oysters were not placed in seawater on arrival for fear of triggering spawning. All oysters were dissected into digestive gland, adductor muscle and gill. In addition, remaining tissues were kept from ten large and ten small oysters. To minimise dissection time, fresh tissues were not weighed before freezing, but will be weighed frozen prior to homogenisation. Tissue sub-divisions are as in

Table 2. As for June 1997, samples were frozen in liquid nitrogen and stored at -70°C.

Table 2. Dissection of tissue samples from July 1997

Sub-task		Proportion of tissue used for each sub-task			
		Digestive gland	Adductor muscle	Gill	Remaining tissues
Proteolytic enzymes	4.3	1/4*	3/4	0	All*
Stress proteins	4.3	0	0	1/2	-
Digestive enzymes	4.4	1/4	0	0	-
Allozymes	5.1	1/2	1/4	0	-
Microsatellites	5.2	0	0	1/2	-

3 RESULTS

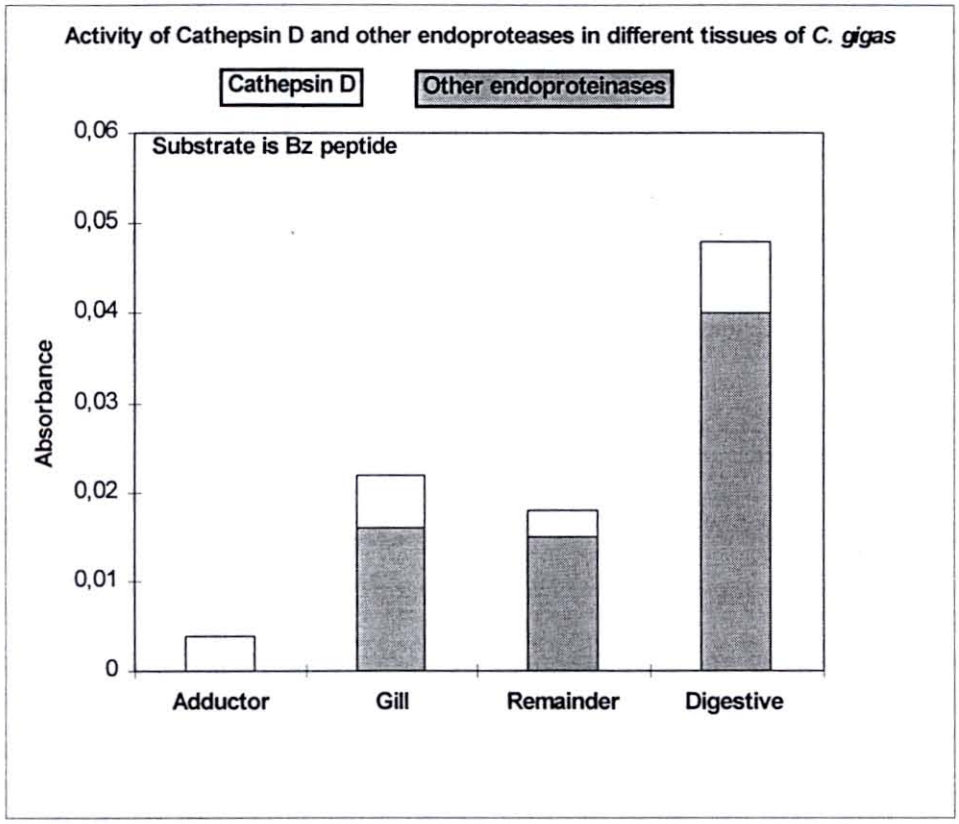
Cathepsin D

Localization of activity

Supernatants for all 4 tissue types were assayed for Cathepsin D activity over 15 mins and at pH 3.3, using both haemoglobin and Bz peptide. The ranking of Cathepsin D activity expressed as absorbance units per 50µl of sample from different tissues was similar for both substrates (Figure 1). When activity in each tissue was recalculated as a percentage of whole-body, measured activities in the digestive gland represented 61% and 50% of whole-body activity, using substrates haemoglobin and Bz peptide as substrates, respectively. Least activity was found in the adductor muscle with 12% for Bz peptide and 1.4% for haemoglobin. The differences between measures may be due to errors associated with low sensitivity of the Bz peptide assay, for absorbances obtained were always less than 0.06 O.D units. Because of these low absorbances, assays to investigate pH profile and time course of activity were performed on digestive gland only.

Endoproteinase activity that was not inhibited by pepstatin, and therefore not due to Cathepsin D, accounted for the majority of activity in digestive gland, gill and remaining tissues, irrespective of which substrate was used (Figure 1). Therefore, compared with haemoglobin, use of Bz peptide as a substrate did not appear to increase specificity for Cathepsin D in *C. gigas*.

Figure 1 : Tissue localisation of Cathepsin D activity in Crassostrea gigas



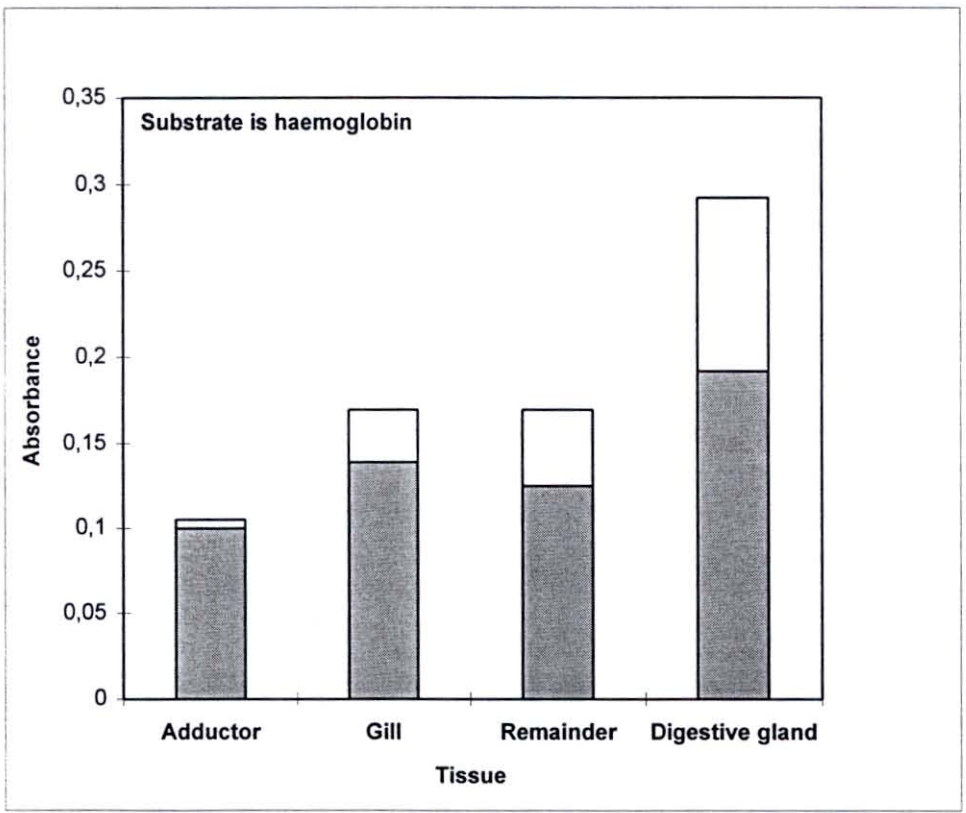
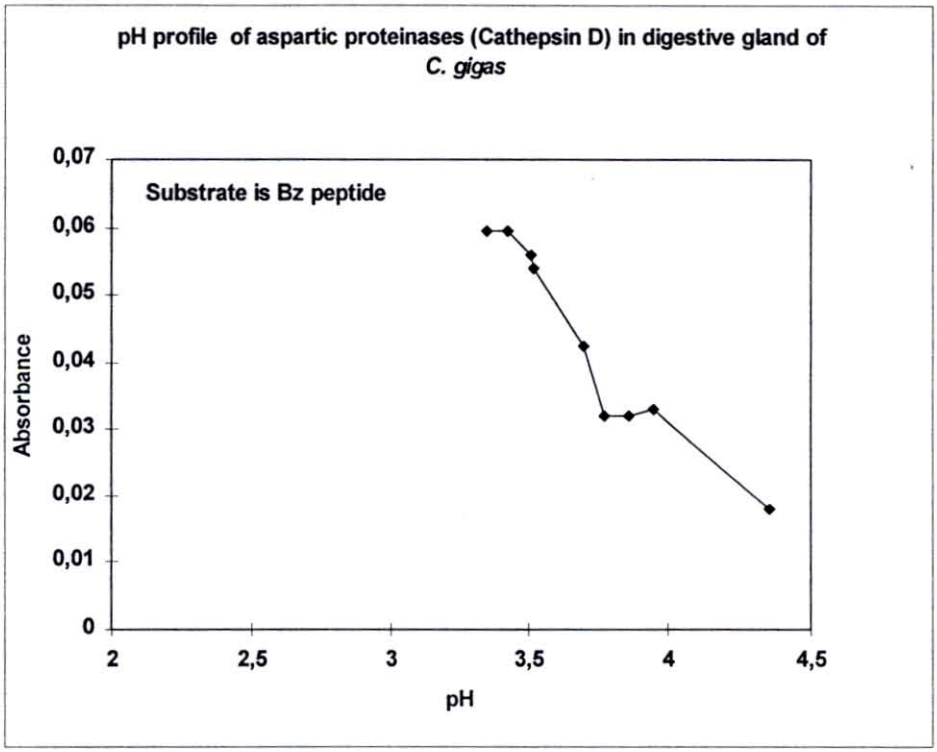


Figure 2 : pH profile of Cathepsin D activity in digestive gland of Crassostrea gigas



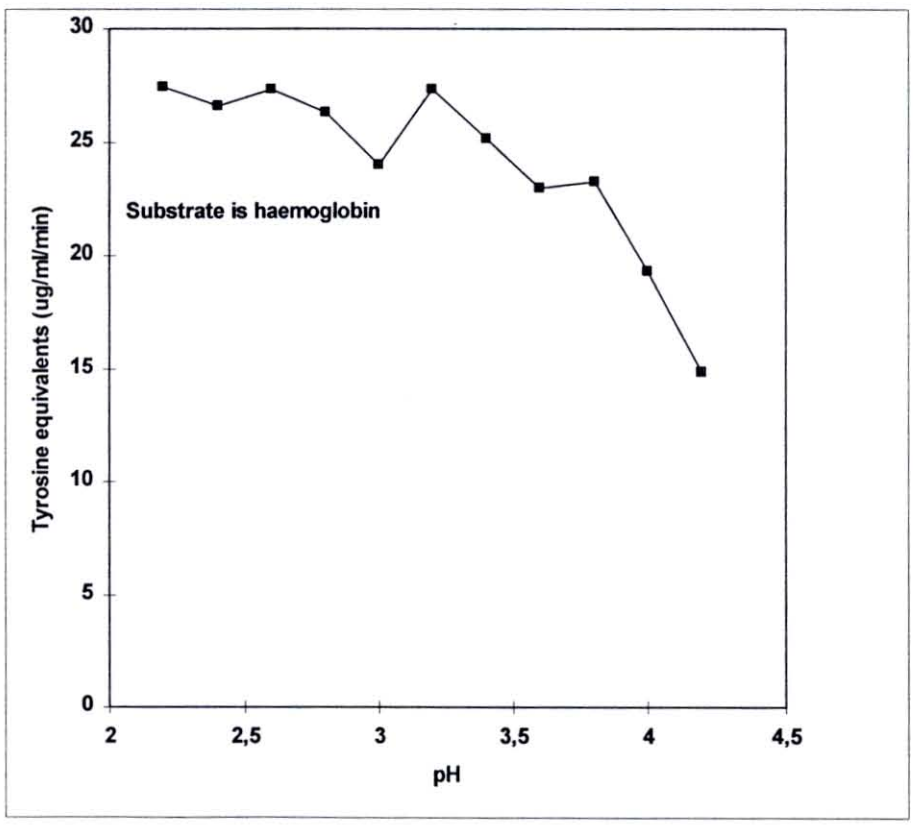
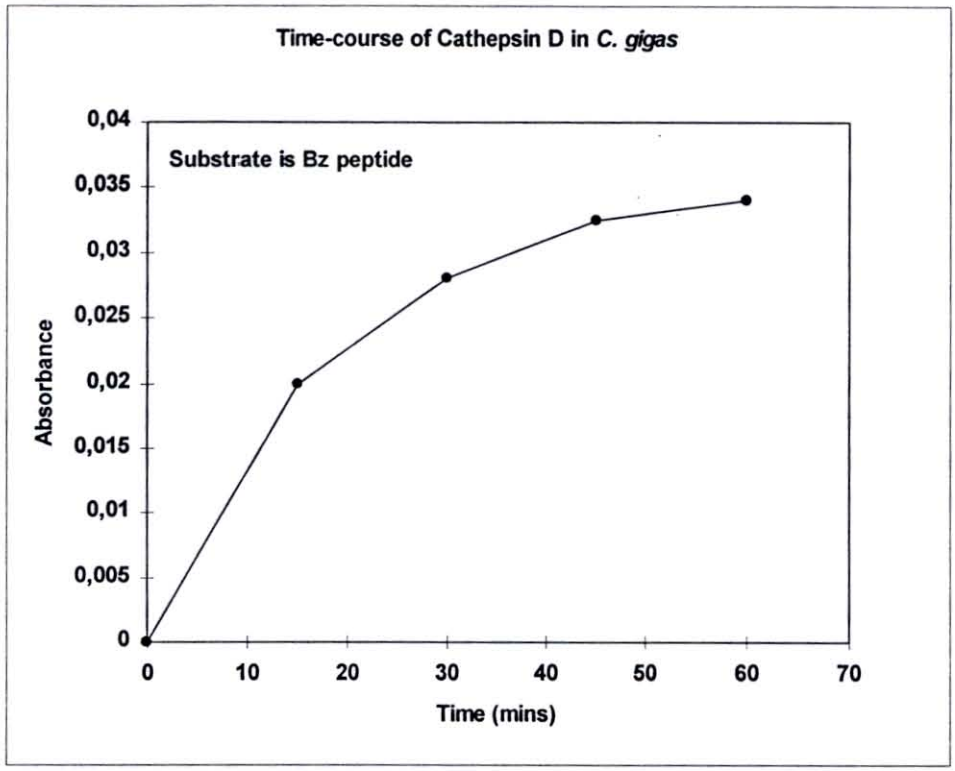
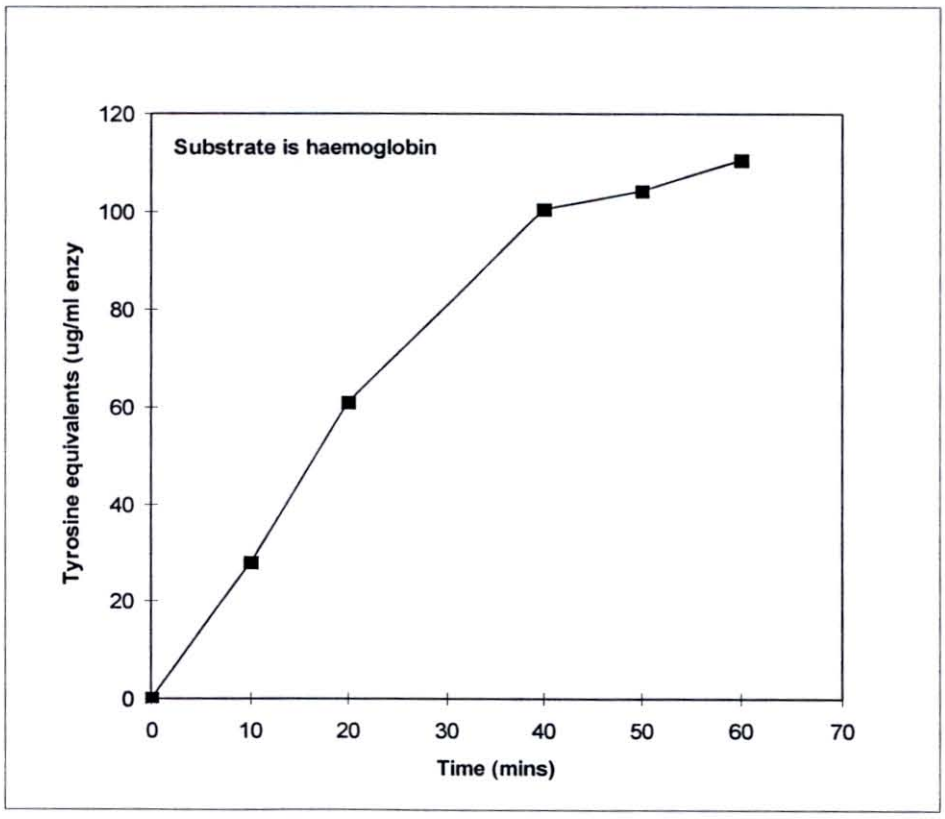


Figure 3 : Time course of Cathepsin D activity in digestive gland of Crassostrea gigas





pH profile

The range of pH tested with the Bz peptide assay varied from 4.5 to 3.3. Lower pH was not possible with 10 mM Formate buffer. Within this range, activity in digestive tissues using Bz peptide was maximal between pH 3.3 and 3.5, declining at higher pH, albeit with a further plateau between 3.7 and 4.0 (Figure 2, top). Previous results for activity within digestive tissues using haemoglobin revealed a similar pattern (Figure 2, bottom).

Time-course

Assays using the Bz peptide were carried out over time intervals from 0 to 60 mins at pH 3.3. Cathepsin D activites were linear for at least 15 minutes (Figure 3, top). This was similar to results obtained with haemoglobin, when activity was linear for 20 minutes (Figure 3, bottom).

4 DISCUSSION

Our previous developmental work on Cathepsin D in *C. gigas* used haemoglobin as a substrate. This substrate is easy to prepare, yields high activities, and it is easy to detect peptide products. However, it is not specific for Cathepsin D, and under acid conditions is also attacked by cysteine proteases, principally Cathepsin L. Assays for Cathepsin D activity using this substrate therefore require a control set of incubations with pepstatin A which is a specific inhibitor for aspartic proteases. The Bz peptide substrate that we have compared with haemoglobin is one of a number of synthetic peptides designed to detect Cathepsin D activity (Agarwal & Rich 1983, Smith & Van Frank 1975). These substrates have limited solubility in acqueous media and are expensive to manufacture, but are of value where they provide a highly specific substrate to identify and localise Cathepsin D activity. The synthetic peptide we tested did not fulfill this requirement, as the majority of enzyme activity detected using Bz peptide was not inhibited by pepstatin. Sensitivity of this assay was also poor. However, the two different assay methods yielded comparable results in terms of tissue distributions, pH curves and time courses. Given this similarity of results, our future analyses of GENEPHYS samples will use haemoglobin, as it offers greater sensivity and ease of use.

This work on Cathepsin D completes our developmental work on the lysosomal proteases. We are currently developing an assay for Calcium-Activated Neutral Proteases (= Calpains), which are one of the best-understood cytosolic systems, where they play an important role in regulatory processes (Croall & DeMartino 1991). Once developed, this collection of enzyme assays will be applied to the stored oysters that have been sampled from each G1 population.

In the meantime, we are also analysing samples from our experiment that measured protein synthesis in the same G1 samples during June 1997. We expect to have all

analyses completed by May 1998, which will place us in a strong position to optimise the design of experiments undertaken later next year.

5 REFERENCES

Agarwal, N. and D.H. Rich. 1983. An improved Cathepsin D substrate and assay procedure. *Analytical Biochemistry*, **130**: 158-165.

Barrett, A.J. and M.F. Heath. 1977. In: *Lysosomes: a laboratory handbook,* edited by J.T. Dingle, Elsevier/ North-Holland biomedical, Amsterdam, 2nd edition, pp. 19-145.

Croall, D.E. and G.N. DeMartino. 1991. Calcium-Activated Neutral Protease (Calpain) system: structure, function and regulation. *Physiological Reviews,* **71 (3)**: 813-847.

Peek, K. 1987. Some biochemical charateristics of isolated adipogranular cells from the mussel *Mytilus edulis* L. pHD thesis, University College of North Wales, Bangor.

Smith, R.E. and R.M.Van Frank. 1975. In: *Lysosomes in biology and pathology VOL 4*, edited by J.T. Dingle & R.T. Dean, North-Holland/American Elsevier, pp. 194-249.

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas":
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°3

CNRS
Centre National de la Recherche Scientifique

Observatoire Océanologique de Villefranche/Mer

Reporting Period:
1st January-31st December 1997

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°3

Total cost to partner n°3 : 120.000 ECU

EC contribution : 60.000 ECU ***% of the total cost :*** 50

Duration : 60 months	***Commencement date :*** 1/1/1996 ***Completion date :*** 31/12/2000

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(IFREMER La Tremblade)

Coordination physiological aspect :
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(IFREMER L'Houmeau)

Participant n°3

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Laboratoire d'Océanographie Biochimique et Ecologie,
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06230 Villefranche sur Mer - FRANCE

Contractor Dr. Catherine THIRIOT

FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1997

Participant n°3 **CNRS UMS 0829**

**Laboratoire d'Océanographie Biochimique et Ecologie,
Observatoire Océanologique de Villefranche sur Mer
06230 Villefranche sur Mer - FRANCE**

Scientific team **Dr. Catherine THIRIOT**
Alexandra LEITAO (CDD)

Objectives :

- Participation in genetical analysis (karyotype analyses, aneuploidy study),
- Data exploitation and synthesis.

Actions in the project :

Task 2	Obtainment and management of the G2 generation
Sub-task 2.1	Selection of the parents G1 and crossbreeding, Identification and assortment of G1 parents with known aneuploidy for the study of the aneuploidy transmission.
Task 5	Genetical analyses
Sub-task 5.4	Karyotypes analysis (leader of this Sub-task, collaboration with Partner 1, IFREMER La Tremblade) a) Perfection of a non-destructive karyological analysis in order to perform animals assortments, b) Karyological analyses in G1 embryos, c) Assessment of the aneuploidy rate in G1 pseudo-cohort and in G2 studied families, d) Study of the aneuploidy transmission in the G2 families,
Task 8	Data processing and results synthesis Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis.
Task 9	General organisation of the project Participation in annual scheduling meetings.

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
2.1		identification of aneuploïds/ PR			FR
5.4	Embryos analysesG1 / PR	Adults analysesG1 / PR	Adults analysesG1 Study of the aneuploidy transmission/ IS	Study of the aneuploidy transmission/ PR	Study of the aneuploidy transmission/ PR
8		Data processing/ PR	Data processing/ IS	Data processing/ PR	Final synthesis/ FR
9	2 meetings with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners

PR = Periodical report
IS = Intermediary Synthesis
FR = Final Report

Research activities during the second reporting period

Task 2 : Obtainment and management of the G2 generation

Sub-task 2.1 : Selection of the parents G1 and crossbreeding

Action : Identification and assortment of G1 parents with known aneuploidy for the study of the aneuploidy transmission.

This objective was not successful in 1997 due to a low mitotic index and a high mortality of the animals tested (see subtask 5.4).

Another experiment is planned in Spring 1998 to sort broodstocks with known aneuploidy by a destructive method.

Task 5 : Genetical analyses

Sub-task 5.4 : Study of aneuploidy and identification of chromosomes.

1. Development of a non destructive method to select broodstocks with a known aneuploidy

The development of a non destructive method for karyological analysis was tested in two experiments. This method consisted in the extraction of a 2-3mm section of the gill from a live animal, after a previous treatment with $MgCl_2$ which allows the opening of the valves by relaxation of the muscles. Thus, the evaluation of aneuploidy of a tagged animal could allow to follow the possible transmission of this phenomenon.

Study of aneuploidy in biopsies of tagged animals (La Tremblade 3-4 December 1996).

Tagging of oysters was performed on 40 animals of two size classes (20 small and 20 large) for the three links (Port des Barques, Bonne Anse, Arcachon). Biopsies of the gills of these animals were made after $MgCl_2$ treatment. The mortality after 48 h was of 2%. Aneuploidy was scored on slide preparations made from 15 animals of each link. The mitosis were absent or scarce in these preparations, so we stopped the study of the remaining animals. This lack of mitosis was probably due to a low mitotic index (non-growing period of winter) and we planed to carry out a new experiment during more favourable conditions (in spring).

Biopsy experiments (La Tremblade 13-18 March 1997).

Biopsies of the gills of the animals (40 per link, 120 in total) tagged in December were made after MgCl2 treatment. This treatment was not totally successful (14 animals remained closed). Slide preparations were performed from 16 animals in Port des Barques, 19 from Bonne Anse and 15 from Arcachon. Few mitosis were observed (Table 1), thus aneuploidy could not be evaluated. Moreover, the mortality was very high after experiment: more than 50% died within two weeks.

This low mitotic index was unexpected for this time of the year. This could be due to various problems: acclimation of animals was not successful, poor conditions of the animals etc...

In conclusion, scoring of aneuploidy in biopsies of gills was not successful due to a low mitotic index. Thus, this non-destructive method can not be used to select broodstocks with a known aneuploidy. Scoring of aneuploidy of broodstocks used for the study of a possible transmission of aneuploidy in the next offspring will be done by a destructive method, i. e. immediately after spawning. This experiment is planned in Spring 1998.

Table 1. Study of aneuploidy in biopsis (March 97)

Port des Barques population

Tagged animal.	Total of mitosis studied	No. of euploid cells	No. of aneuploid cells		
N°		2n=20	2n=19	2n=18	2n=17
3	0	0	0	0	0
4	1	1	0	0	0
5	2	2	0	0	0
6	0	0	0	0	0
10	6	6	0	0	0
11	0	0	0	0	0
14	2	2	0	0	0
17	0	0	0	0	0
38	2	1	0	1	0
36	0	0	0	0	0
34	3	3	0	0	0
32	3	3	0	0	0
33	0	0	0	0	0
31	0	0	0	0	0
27	0	0	0	0	0
22	0	0	0	0	0

Bonne Anse population

Tagged animal.	Total of mitosis studied	No. of euploid cells	No. of aneuploid cells		
N°		2n=20	2n=19	2n=18	2n=17
6	7	4	2	1	0
7	8	6	0	2	0
8	0	0	0	0	0
12	2	2	0	0	0
13	0	0	0	0	0
18	0	0	0	0	0
40	3	3	0	0	0
36	3	3	0	0	0
38	6	6	0	0	0
14	2	2	0	0	0
10	9	7	1	1	0
3	1	1	0	0	0
28	13	12	1	0	0
39	1	0	1	0	0
27	4	4	0	0	0
21	0	0	0	0	0
34	0	0	0	0	0
24	0	0	0	0	0
26	0	0	0	0	0

Arcachon population					
Tagged animal.	Total of mitosis studied	No. of euploid cells	No. of aneuploid cells		
N°		2n=20	2n=19	2n=18	2n=17
4	0	0	0	0	0
10	0	0	0	0	0
16	4	4	0	0	0
8	6	5	1	0	0
21	0	0	0	0	0
23	1	1	0	0	0
31	0	0	0	0	0
28	5	5	0	0	0
18	0	0	0	0	0
2	4	4	0	0	0
33	4	2	2	0	0
36	2	2	0	0	0
17	2	1	0	1	0
3	0	0	0	0	0
29	0	0	0	0	0

2. *Estimation of aneuploidy*

Comparative study of aneuploidy in the three links (Port des Barques, Bonne Anse, Arcachon).

Animals of two size classes from each link were acclimated at La Tremblade during five days then treated and fixed for chromosome study at La Tremblade (7-8 October 1996). Chromosome preparations and scoring of aneuploidy were made at Villefranche sur Mer in February 97. The results (Tables 1, 2, 3) are given for 13 small and 10 large animals from Port de Barques, 12 small and 16 large animals from Bonne Anse and 11 small and 10 large animals from Arcachon. 30 mitosis were scored in most of the animals, but in some animals from Arcachon, due to the low number of available mitosis counting of the 30 mitosis was not possible. In total, 72 animals and 2037 mitosis were studied.

The comparison between the percentage of aneuploidy in the three links was evaluated on a same number of animals chosen at random in two size classes, i.e.ten small animals from Port des Barques (25%), Bonne Anse (25%) and Arcachon (30%) and in ten large animals from Port des Barques (18%), Bonne Anse (19%) and Arcachon (22%). These percentages confirm the correlation between somatic aneuploidy and growth rate (Thiriot-Quiévreux et al 1986, 1992)

Looking at all animals studied, the total percentage of aneuploidy in the links (Port des Barques 23%, Bonne Anse 22%, Arcachon 25%) is slightly lower than the one observed last year in the pseudo-cohorts (27%, 34%, 33%, 28% respectively), but still remains relatively high compared to previous results on other populations of *Crassostrea gigas*. Using an ANOVA (one factor), the frequencies of aneuploidy of the three links were not significantly different at p=0.05.

Table 2: Study of aneuploidy in Link 2: Port des Barques

Animal #	Size of the animal	Total of mitosis studied	N° of euploid. cells	No. of aneuploid cells			
			2n=20	2n=19	2n=18	2n=17	total
3	small	30	23	2	4	1	7
4	small	30	23	2	3	2	7
5	small	30	22	4	2	2	8
6	small	30	25	4	1	0	5
7	small	30	22	4	4	0	8
8	small	30	21	5	2	2	9
9	small	30	22	5	2	1	8
10	small	30	22	4	3	1	8
11	small	30	22	4	1	3	8
12	small	30	22	2	5	1	8
13	small	30	20	7	3	0	10
1	small/M	30	20	3	5	2	10
2	small/M	30	22	4	2	2	8
14	large	30	22	4	2	2	8
15	large	30	27	2	1	0	3
16	large	30	25	3	2	1	5
17	large	30	26	2	2	0	4
18	large	30	25	1	4	0	5
19	large	30	25	1	4	0	5
20	large	30	24	4	2	0	6
21	large	30	25	1	4	0	5
22	large	30	25	4	0	1	5
23	large	30	23	5	1	1	7

small/M: small to medium animals

Estimation of aneuploidy		
Total percentage of aneuploidy	23%	N=23
Aneuploidy in **small** animals	**25%**	**N=10**
Aneuploidy in small and small/M animals	27%	N=13
Aneuploidy in **large** animals	**18%**	**N=10**

Table 3. Study of aneuploidy in Link 3: Bonne Anse

Animal #	Size of the animal	Total of mitosis studied	N° of euploid. cells	No. of aneuploid cells			
			2n=20	2n=19	2n=18	2n=17	total
1	small	30	22	6	1	1	8
2	small	30	25	3	1	1	5
3	small	30	21	5	3	1	9
4	small	30	22	5	3	0	8
5	small	30	19	6	4	1	11
6	small	30	24	5	1	0	6
7	small	30	24	5	0	1	6
8	small	30	20	9	0	1	10
9	small	30	25	2	3	0	5
10	small	30	24	5	1	0	6
11	small/M	30	21	2	4	3	9
12	mediun	30	23	5	2	0	7
13	M/large	30	23	4	3	0	7
14	large	30	24	5	1	0	6
15	large	30	22	5	3	0	8
16	large	30	22	5	2	1	8
17	large	30	22	5	3	0	8
18	large	30	25	4	1	0	5
19	large	30	27	1	1	1	3
20	large	30	24	3	1	2	6
21	large	30	24	5	1	0	6
22	large	30	25	2	2	1	5
23	large	30	25	3	2	0	5
24	large	30	24	2	2	2	6
25	large	30	23	5	1	1	7
26	large	30	27	1	1	1	3
27	large	27	24	1	1	1	3
28	large	30	22	1	4	3	8

M/large: medium to large animal

small/M: small to medium animals

Estimation of aneuploidy		
Total percentage of aneuploidy	22%	N=28
Aneuploidy in **small** animals	**25%**	**N=10**
Aneuploidy in small, small/M and medium animals	26%	N=12
Aneuploidy in **large** animals	**19%**	**N=10**
Aneuploidy in large animals	19%	N=16

Table 4. Study of aneuploidy in Link 4: Arcachon

Animal #	Size of the animal	Total of mitosis studied	N° of euploid. cells	No. of aneuploid cells			
			2n=20	2n=19	2n=18	2n=17	total
1	small	20	13	3	2	2	7
2	small	30	24	2	3	1	6
3	small	30	23	2	3	2	7
4	small	14	8	1	3	2	6
5	small	10	9	1	0	0	1
6	small	14	10	1	1	2	4
7	small	30	17	8	4	1	13
8	small	30	20	5	5	0	10
9	small	28	20	6	2	0	8
10	small	30	22	6	2	0	8
11	small/M	20	17	0	2	1	3
12	large	30	24	2	3	1	6
13	large	18	13	2	2	1	5
14	large	23	19	1	2	1	4
15	large	30	24	4	1	1	6
16	large	30	23	6	1	0	7
17	large	20	12	1	4	3	8
18	large	30	23	5	0	1	6
19	large	17	14	2	1	0	3
20	large	30	25	1	3	1	5
21	large	26	20	2	3	1	6

small/M: small to medium animals

Estimation of aneuploidy		
Total percentage of aneuploidy	25%	N=21
Aneuploidy in **small** animals	**30%**	**N=10**
Aneuploidy in small, small/M and medium animals	29%	N=11
Aneuploidy in **large** animals	**22%**	**N=10**

Study of aneuploidy in a control population

Due to the high percentage of aneuploidy observed in the oysters originating from La Tremblade, a control population was studied.

Broodstock oysters originating from Scotland were used for reproduction at the Argenton hatchery (IFREMER Station in Bretagne), juveniles were reared at Bouin (IFREMER station on the French Atlantic coast).

Initially, a few animals (N=11) were sent to Villefranche (April 1997) in order to make chromosome preparations and study aneuploidy. Table 5 shows the result of this study. Five (out 11 animals) only included normal cells (with 2n= 20). The total percentage of aneuploidy (2n= 19, 18 and 17) was of 3%, i. e. very low. Two animals showed cells with 2n=21. Looking at 18 karyotypes of these abnormal animals, three hypotheses were suggested: (i) occurrence of trisomy on pair 10 (11 out 17 karyotypes), (ii) occurrence of a small supernumerary chromosome (6 out 18 karyotypes), (iii) occurrence of a large supernumerary chromosome (2 out 25 cells observed).

Table 5. Study of aneuploidy in a control population (shell size 3.5 to 4.7 cm) (April 1997)

Animal #	Total of mitosis studied	N° of euploid cells 2n=20	No. of aneuploid cells 2n=19	2n=18	2n=17	total	2n=21
1	30	30					
2	30	30					
3	30	30					
4	25	23	1	1		2	
5	30	30					13
6	30	29		1		1	
7	17	15	2			2	
8	30	30					
9	30	30					
10	29	25	1		3	4	
11	22	21		1		1	12
Estimation of aneuploidy							
Total percentage of aneuploidy					3%		N=11

Further study was initiated due to the low percentage of aneuploidy observed in this small sample set. Subsequently, 30 animals (15 small and 15 large) were sent to Villefranche in September 1997. Animals were treated and fixed for chromosome study. Chromosome preparations and aneuploidy study are in progress.

Identification of oyster chromosomes by banding techniques

"G-bands" were performed using ASG technique in *C. virginica* and *O. edulis* acclimated at La Tremblade in spring 97, then in *C. gigas*, acclimated in September 97. These bands allowed us to identify the different pairs of a karyotype which showed a specific pattern. G-band patterns were seen in *C.virginica* (Fig. 1), in Ostrea edulis (Fig. 2), and in *C. gigas* (Fig. 3). These patterns were repetitive in 19 mitosis of *C. virginica*, 14 in *O. edulis* and 10 of *C. gigas*. In some chromosomes, e.g. chromosome pairs n° 8 and 10 in *C. gigas*., repetitive bands were not observed in enough mitosis to be sure of the results.

"R-bands" were also performed (Dutrillaux & Couturier 1981). These bands are reverse

bands of G-bands and permit a complementary diagnostic for identification of chromosomes. Preliminary results (Fig. 1, 2 and 3) were obtained on 5 mitosis of *C. virginica*, 5 mitosis of *O. edulis* and 5 in *C. gigas*.

It should be emphasized that the results on G and R-bands are the first results obtained in oysters and hence these findings are of great interest.

The location of NOR (nucleolus organizer regions) has been observed on pair no. 10 of *C. gigas* (confirming previous results), on pairs n° 7, 9 and 10 of *O. edulis* and on pairs n° 1 and 5 in *C. virginica*.

Our first results on C-bands showed positive C-bands on pairs n° 1, 5 and 7 of *O. edulis*, on pairs 3, 5 and 7 of *C. virginica*. *C. gigas* did not give positive results.

3. Conclusion

Aneuploidy in biopsies of oysters made in December 1996 and March 1997 could not be evaluated because of the lack of mitosis. This could be due to a low mitotic index at the time of the year these samples were taken (December) or other condition factors in the March samples. Scoring of aneuploidy of broodstocks used for the study of a possible transmission of aneuploidy in the next offspring will be done by a destructive method, i. e. immediately after spawning. This experiment is planned in Spring 1998.

The comparison between the percentage of aneuploidy in ten small animals from each link and in ten large animals confirms the correlation between somatic aneuploidy and growth rate. Looking at all animals studied, the total percentage of aneuploidy in the links is slightly lower than the ones observed in the pseudo-cohorts studied in 1996. However these results are still relatively high compared to previous results on other populations of *Crassostrea gigas*.

The preliminary results on a control population were interesting because of the low percentage of aneuploid observed.

Identification of chromosome pairs of karyotypes of three species of oysters (*C. gigas, C. virginica and O. edulis*) was successful using G-band and R-band techniques.

Figure 1 : *Crassostrea virginica*

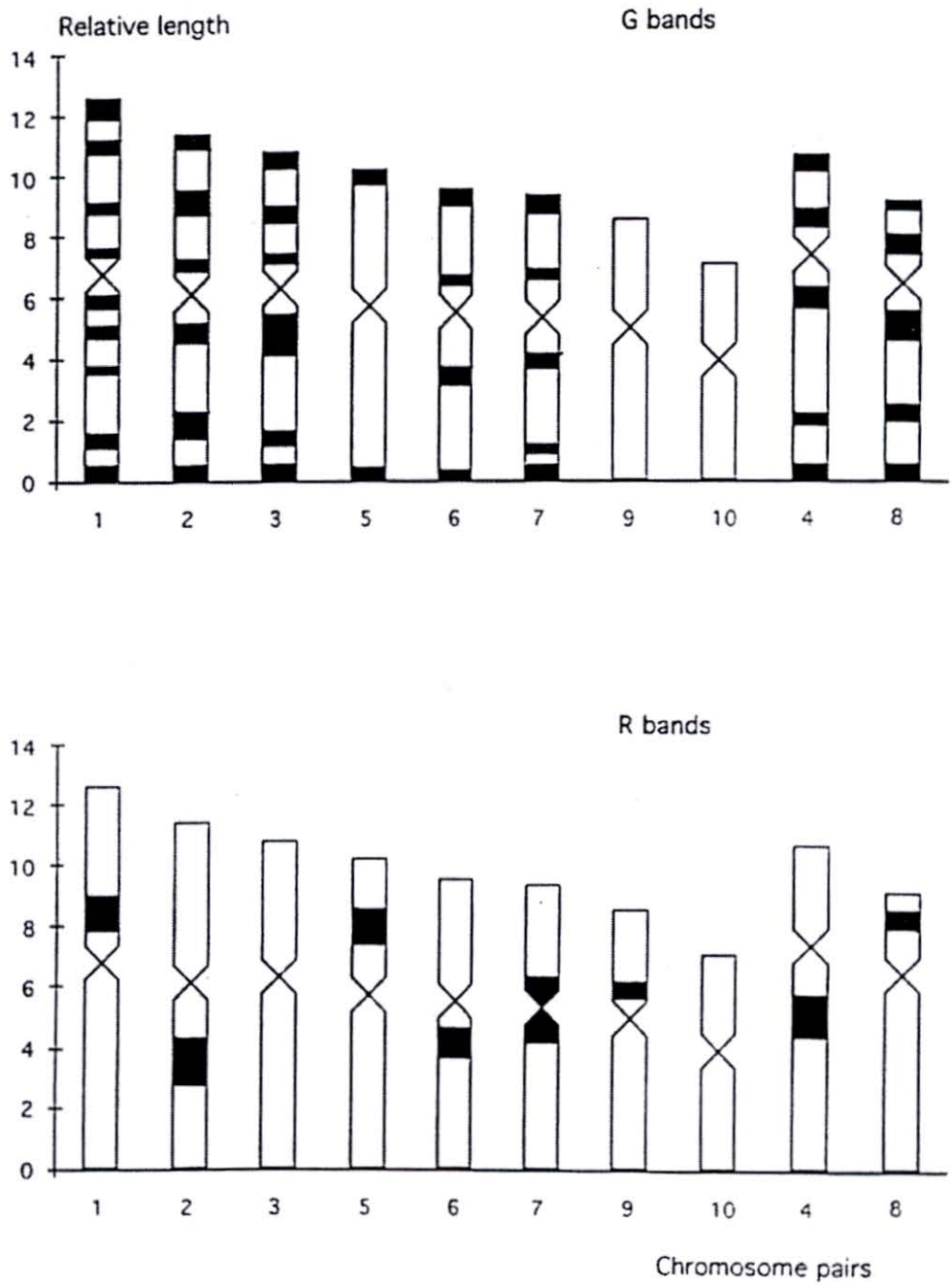


Figure 2 : *Ostrea edulis*

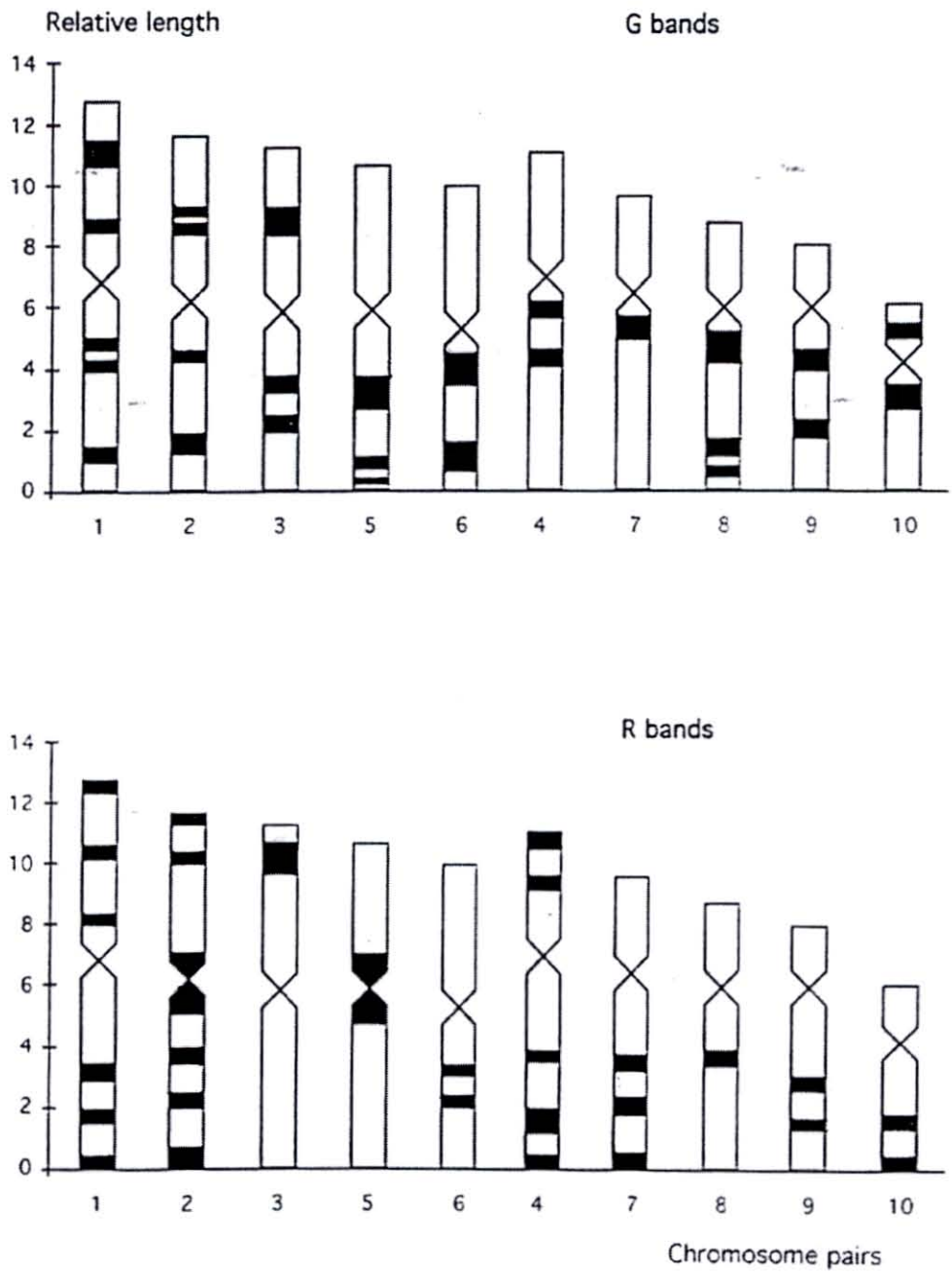
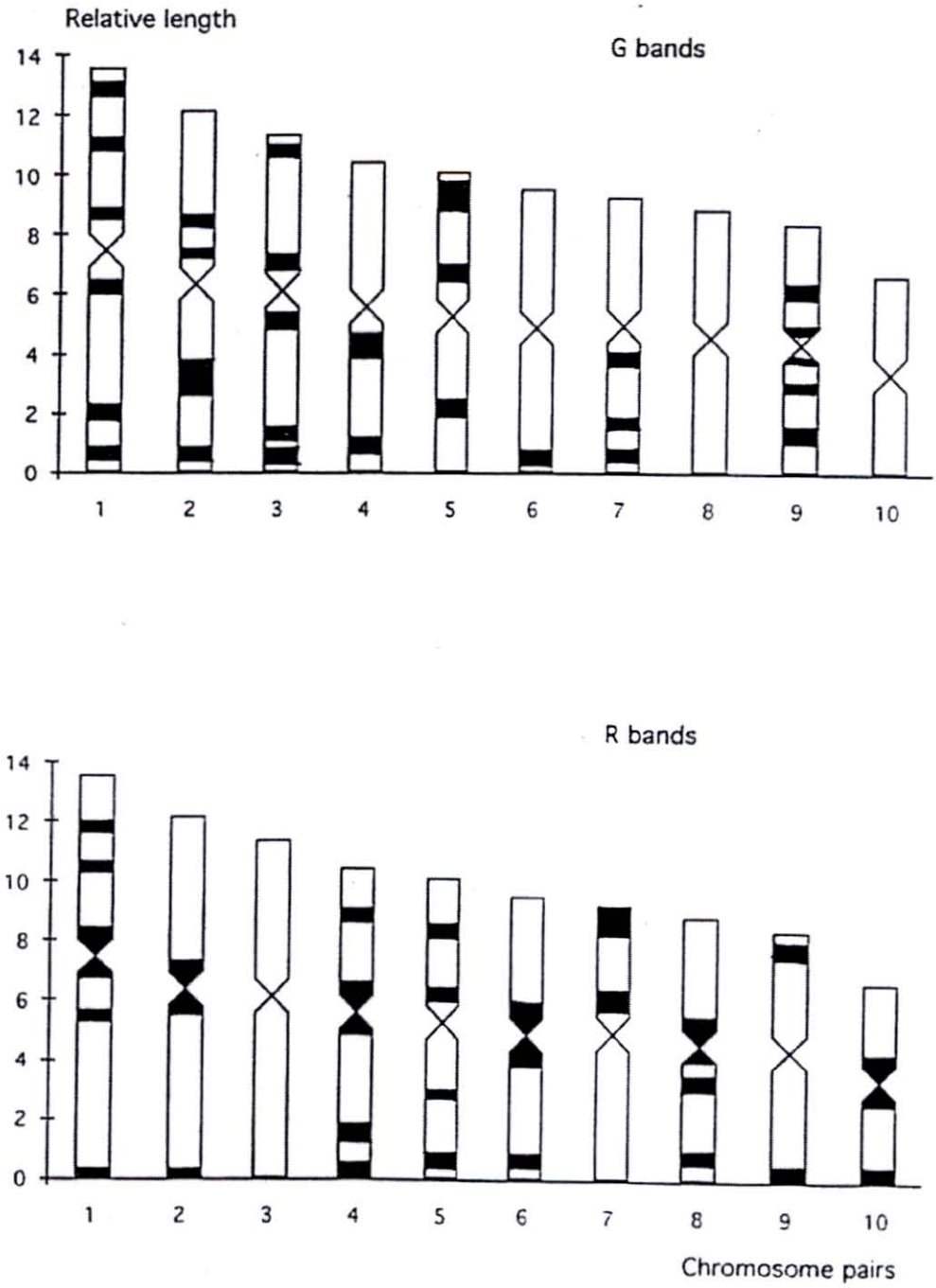


Figure 3 : *Crassostrea gigas*



4. *Planned research activities in 1998*

- Scoring of aneuploidy in animals (September 97) of the control population.
- Scoring of aneuploidy in animals where individual growth rate was studied by partner 1.
- Study of aneuploidy (destructive method) of broodstoks G1 used for G2.
- Study of aneuploidy of G2 offspring.
- Identification of chromosomes by banding techniques.

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°4

UCG
University College Galway

Departement of Zoology

Reporting Period:
1st January-31st December 1997

ANNEX III
Individual Progress Report
Title page to participant n°4

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°4

Total cost to partner n°4 : 150.000 ECU

EC contribution : 150.000 ECU ***% of the total cost :*** 100

Duration : 60 months	***Commencement date :*** 1/1/1996 ***Completion date :*** 31/12/2000

EC contact : DG XIV / C.2 (Fax : (+32-2)295.78.62

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Coordination genetic aspect :
Dr Pierre BOUDRY
(IFREMER La Tremblade)

Coordination physiological aspect :
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Contractor Dr. Noël WILKINS

FAIR PL. 95.421

Individual Progress Report

for the period from

1st January to 31st December 1997

Participant n°4 **University College Galway**
Department of Zoology
Ireland

Scientific team **Pr. N. P. WILKINS**
Dr. R. POWELL
Pr. J.A. HOUGHTON
S. HUBERT (PhD)
G. RAFFERTY (PhD)

Objectives :

- Participation in genetical analyses (allozymes, aneuploidy study),
- Research of chromosomal markers,
- Data exploitation and synthesis.

Actions in the project :

Task 5	Genetical analyses
Sub-task 5.1	Allozymes realisation of the whole sub-task by the Partner.
Task 7	Research of chromosomal markers Realisation of the whole Task by the Partner.
Task 8	Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis.
Task 9	General organisation of the project Participation in annual scheduling meetings.

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
5.1	Lab analyses G0 / PR	Lab analyses G1 / PR	Lab analyses G1 / IS		FR
7	Perfectionment of the technique / PR	Perfectionment and application / PR	Aneuploïds characterisatio n / IS	Aneuploïds characterisatio n / PR	Aneuploïds characterisatio n / PR
8		Data processing/ PR	Data processing/ IS	Data processing/ PR	Final synthesis/ FR
9	2 meetings with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners

PR = Periodical report

IS = Intermediary Synthesis

FR = Final Report

Research activities during the second reporting period

Task 5 : Genetical analyses

Sub-task 5.1 : Allozymes

1 Introduction

In recent years, numerous electrophoretic studies of wild populations of bivalves have revealed a relationship between heterozygosity and growth rate. Positive association between allozymic heterozygosity and fitness characters have been observed in a variety of marine molluscs including *Crassostrea virginica* (Singh & Zouros, 1978, 1980 ; Zouros *et al*, 1980, 1983), *Crassostrea gigas* (Fujio, 1982), *Mytilus edulis* (Koehn & Gaffney, 1984 ; Diehl &Koehn, 1985, Rodhouse *et al*, 1986 ; Zouros *et al* 1988 ; Gaffney, 1990), *Mulinia lateralis* (Garton *et al*, 1984 ; Koehn *et al* 1988, Gaffney et al, 1990), *Placopecten magellanicus* (Foltz & Zouros, 1984 ; Pogson & Zouros, 1994), *Ostrea edulis* (Alvarez, 1989), *Mytilus galloprovincialis* (Gardner & Skibinski, 1988). But some opposite examples also occur, sometimes with same species e.g. *Mytilus edulis* (Gosling, 1989), *Ostrea edulis* (Saavedra, 1996), *Placopecten magellanicus* (Volckaert & Zouros, 1989), *Crassostrea gigas* (Zouros *et al*, 1996). In some species e.g *Mytilus edulis* (Mallet *et al*, 1986 ; Beaumont, 1991), *Crassostrea virginica* (Foltz & Chatry, 1986), *Mulinia lateralis* (Gaffney & Scott, 1984) *Crassostrea gigas* (Thiriot-Quievreux *et al*,1992) no correlation was observed between heterozygosity and growth in restricted or in pair mating, although such correlations have been regularely found in wild populations.

Few data are available about the correlation between heterozygosity and growth rate in *Crassostrea gigas* (Zouros, 1996). Most studies have been based on samples from natural populations or from hatchery populations where nothing is known about the parentage (Fujio, 1982 ; Osaka, 1982 ; Gosling, 1982 ; Lucas, 1983 ; Hedgecock, 1986, Moraga, 1989).

Parents from wild populations were crossed to obtain 3 offspring families which were then reared in the hatchery. Heterozygosity and growth were analysed to determine whether they are correlated in these families and the inheritance of the alleles was investigated.

2 Materials and Methods

Oysters *Crassostrea gigas* were sampled from 3 different areas viz. Seudre Estuary, Port des Barques and Bonne Anse (France). The GENEPHYS parents (Go) were drawn from 30 individuals from each population. The offsprings oysters (G1) were ongrown in La Tremblade. For the analysis, juvenile G1 oysters were weighed, then sacrified. Adductor muscle and digestive gland were removed and immediatly frozen at -70°C. The tissues were sent to Galway where there were stored at -70°C until electrophoresis. Electrophoretic separation of allozymes was done on horizontal starch gels. The electrophoretic conditions are presented in table 1. The G0 parents and the G1 juveniles were analysed on the same gels.

Enzyme Name	Abbreviations	Ec Enzyme No	Electrophoretic buffer	Tissue	Loci screened
Phosphoglucomutase	PGM	2.7.5.1	Tills Tris Maleic 7.4	Muscle	PGM-2
Leucine Amino Peptidase	LAP		Tills Tris Maleic 7.4	Muscle	LAP-2
Esterase	EST	3.1.1.2	Ridgeway	Digestive Gland	EST-3
Esterase D	ESTD		Tills Tris Maleic 7.4	Digestive Gland	ESTD-2, ESTD-3
Isocitrate dehydrogenase	IDH	1.1.1.42	Tris Citrate (TC) 7.0	Digestive Gland	Idh-1, Idh-2
Malate dehydrogenase	MDH	1.1.1.37	Tris Citrate (TC) 7.0	Digestive Gland	Mdh-1, Mdh-2
Glucose-6-Phosphate Isomerase	GPI	5.3.1.9	Ridgeway	Muscle	GPI-1

Table 1 : Summary details of electrophoretic conditions for each of the loci

Expected genotypes for the offspring of each family were calculated from the parental genotypes as follows : the allele frequencies were calculated from the genotypes of the males and the females separately in order to estimate the allele frequencies in the sperm and the eggs. Expected offsprings genotypes were then calculated from the gamete frequencies, assuming that all males and females make equal contribution to the offspring generation (first progress report, GENEPHYS). Departures of offsprings genotype proportions from the expected values were tested using the χ^2 goodness-of-fit test.

Correlation between total weight and the number of heterozygous loci in each

individual was calculated for all individuals .

Student's t -test was used for the comparison of mean shell weights of homozygotes and heterozygotes for each locus. Anova was performed to compare different genotype distribution within loci.

The Hardy Weinberg equilibrium was tested using a Markov chain method without bias to give the exact probability value of the comparison of observed and expected genotype proportions within the parental and within the offspring generations. The P value is associated with Ho (ie Hardy Weinberg equilibrium.). Data were analysed using sotfware BIOSYS-1 (release1.7; Swofford & Selander, 1989), GENEPOP (release 1.2, Raymond and Rousset,1995), Statview and Excel.

3 Results

3.1 Allelic frequencies and genotypic proportions.

Allelic frequencies of the Go parents and the G1 juveniles for the 3 populations **Ports des Barques, Arcachon, Bonne Anse** are presented in table 2. No new alleles (ie alleles not observed in the G0 parents) were found in the juveniles, so it seems there are no foreign individuals contaminating the experimental families.

However, there were significant differences in some alleles between the parents and the offspring. Parental allele frequencies are theorical values assuming that all the parents contribute gametes to the fertlization pool. If some parents do not contribute at all, or contribute lower proportions of gametes than others,then the effective allele frequencies in the parents will be different from the actual value indicated here. This could explain the differences observed between the parents and the offspring.

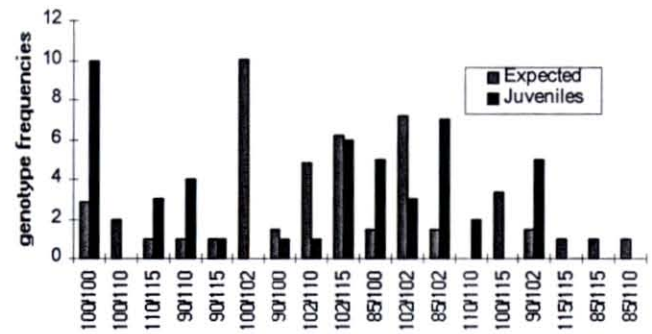
The genotype frequencies for each locus are represented in figures 1,2,3. In the **Port des Barques** populations, no unexpected genotypes were found in juveniles compared with those expected from the parental allele frequencies (figure 1). The $\chi 2$ goodness-of-fit test shows that genotype proportions of juveniles were significantly different from the parent (Table3) for all loci.

In the **Arcachon** population, some genotypes were unexpected like Est1$^{102/102}$, Est1 $^{104/104}$, Idh-2 $^{90/90}$ and Pgm $^{110/110}$ (figure 2). All were homozygotes and it is possible that these are misreadings. They involved two individuals for Est1, one for Idh2 and one for Pgm. The $\chi 2$ goodness-of-fit test (table 3) shows that at 2 loci (Lap and est1) there was no significant difference between observed and expected genotypes. The loci Gpi is only different at a probability of 0.05 and the locus EstD-2 is only different at a probability of 0.01.

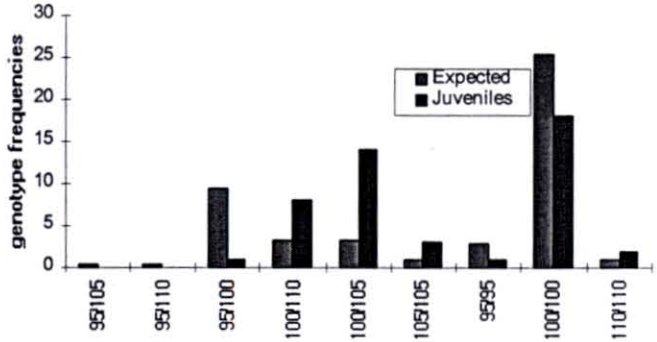
In the **Bonne Anse** population, no unexpected genotypes were found (figure 3). The $\chi 2$ goodness-of-fit test shows that values at Idh2 are only different at a probability of 0.1 (table 3).

	Arcachon		Port des Barques		Bonne Anse	
Locus	Juveniles	Parents	Juveniles	Parents	Juveniles	Parents
PGM						
(N)	45	10	50	10	49	10
85			.020	.050		
90			.130	.050		
95	.044	.200	.360	.200	.153	.100
100	.844	.600	.120	.400	.490	.400
102	.033	.050	.240	.150	.184	.150
110	.078	.150	.130	.150	.102	.250
115					.051	.050
LAP						
(N)	48	9	48	10	50	10
90	.042	.050				
95	.719	.650	.030	.167	.060	.200
100	.042	.050	.590	.722	.870	.600
105	.198	.200	.230	.056	.050	.200
110		.050	.150	.056		
GPI						
(N)	48	10	50	10	50	10
90	.031	.150			.040	.050
100	.927	.800	1.000	1.000	.880	.700
110	.042	.050			.080	.250
Mdh-1						
(N)	50	10	49	10	50	10
100	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-2						
(N)	50	10	49	10	50	10
100	1.000	1.000	1.000	1.000	1.000	1.000
Idh-1						
(N)	50	10	49	10	50	10
100	1.000	1.000	1.000	1.000	1.000	1.000
Idh-2						
(N)	48	10	49	10	49	10
85	.188	.050	0	.050		
90	.031	.050	.061	.100		
95	.698	.800	.061	.300	.235	.250
100	.083	.050	.612	.450	.745	.700
110			.276	.100	.020	.050
Est-1						
(N)	50	10	48	10	50	10
95					.020	.050
98	.220	.200	.115	.100	.550	.300
100	.630	.650	.552	.550	.350	.500
102	.100	.100	.292	.250	.080	.150
104	.050	.050.	.042	.100		
EstD-2						
(N)	48	10	43	10	49	10
100	.188	.300	.488	.650	.265	.350
105	.750	.650	.512	.350	.694	.500
110	.063	.050			.041	.150
EstD-3						
(N)	48	10	47	10	50	10
95	.0	.050	.021	.100		
100	.948	.850	.979	.900	1.000	1.000
105	.052	.100				

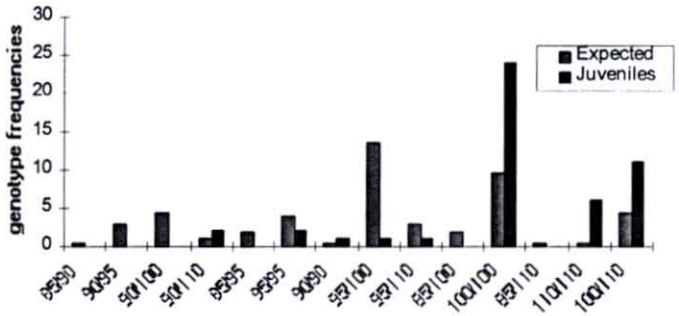
Table 2 : Allelic frequencies of parents and juveniles in the 3 populations.



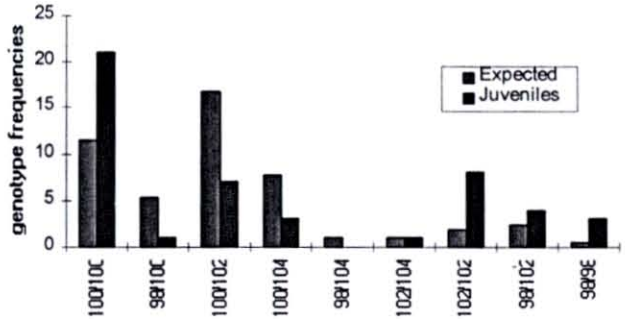
PGM locus



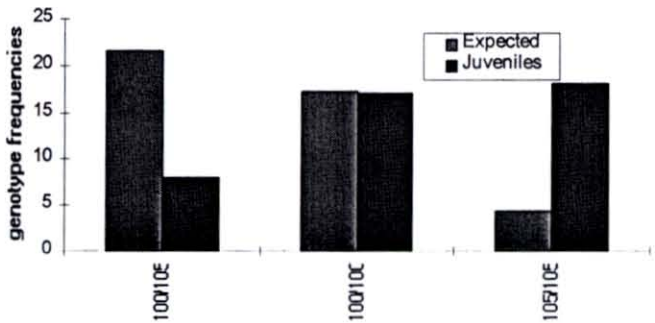
Lap Locus



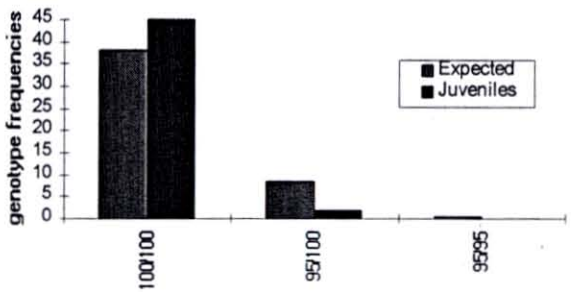
Idh-2 locus



Est-1 locus

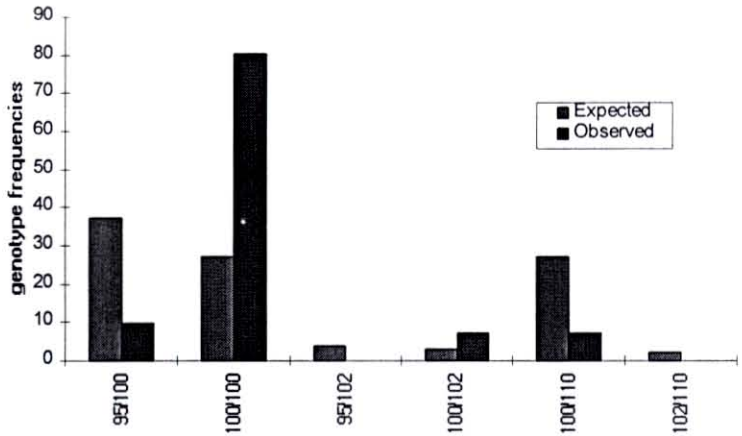


EstD-2 locus

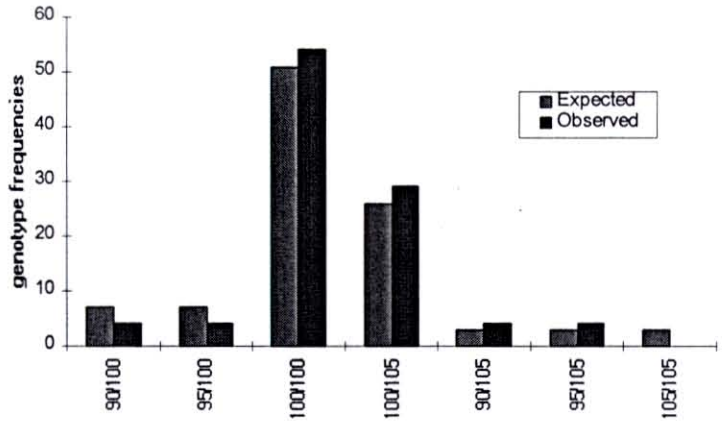


EstD-3 locus

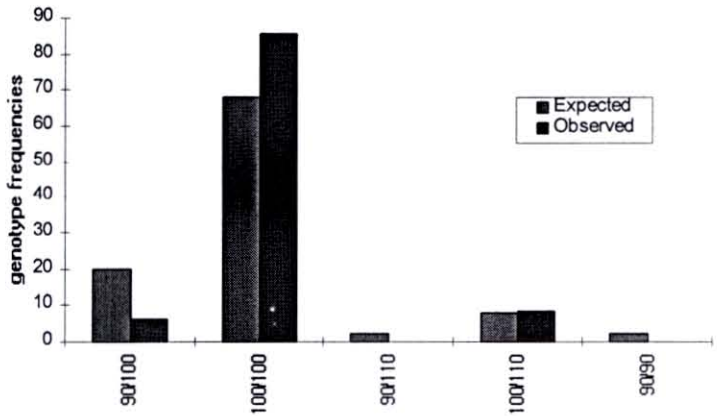
*Figure 1 : Genotype frequencies observed in juveniles compared with the genotype frequencies expected from the parental allele frequencies at the **Port des Barques** populations.*



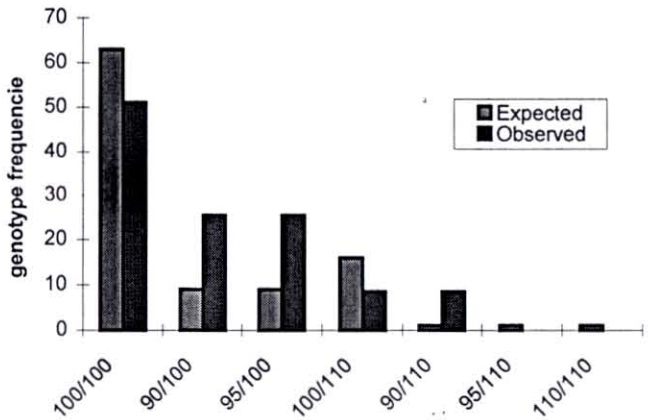
Pgm Locus



Lap locus

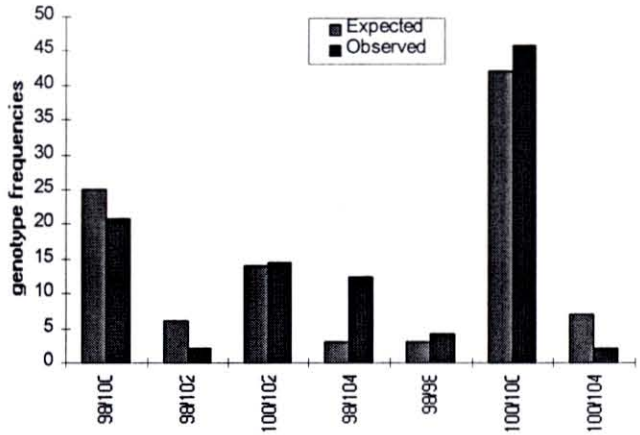


Gpi locus



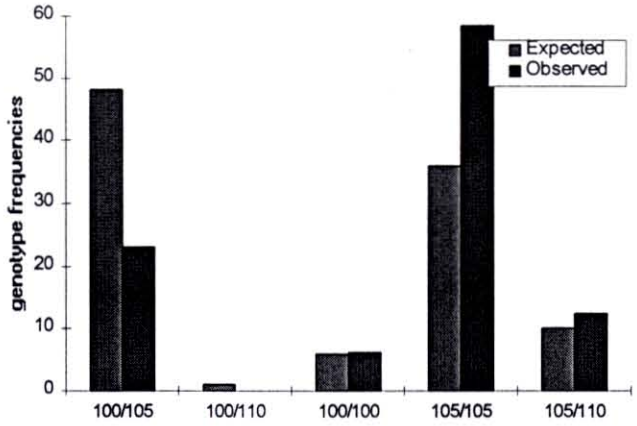
Idh-2 locus

Figure 2 : Genotypic frequencies observed in juveniles compared with the genotype frequencies expected from the parental allele frequencies at the ***Arcachon*** *population.*

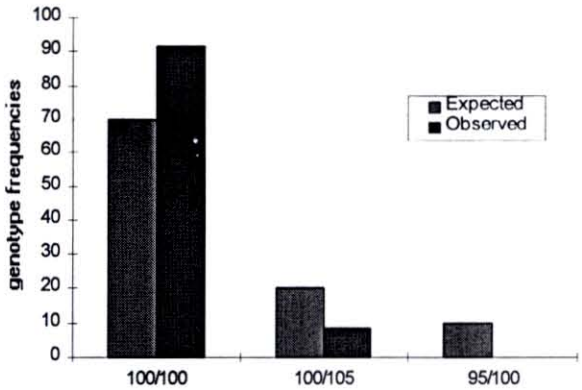


Est-1

locus

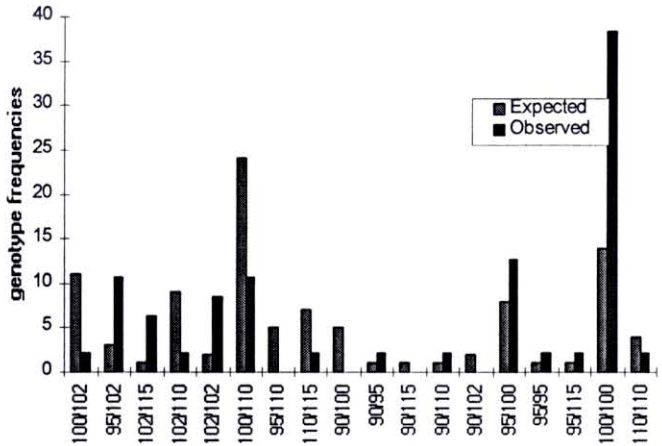


EstD-2 locus

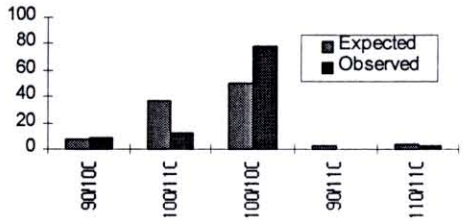


EstD-3 locus

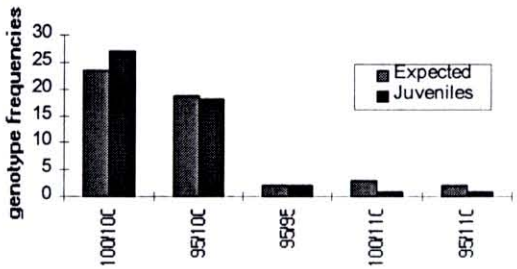
*figure 2 (continued): Genotypic frequencies observed in juveniles compared with the genotype frequencies expected from the parental allele frequencies at the **Arcachon** population.*



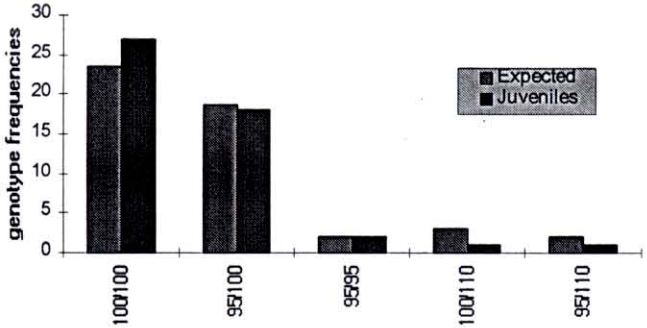
PGM locus



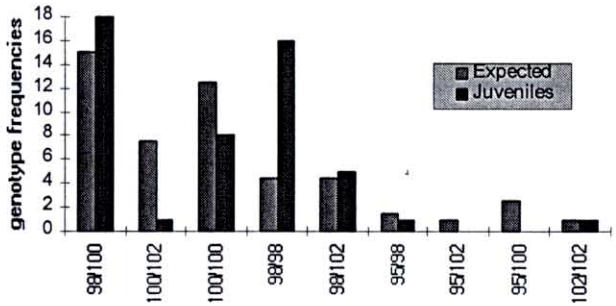
Gpi Locus



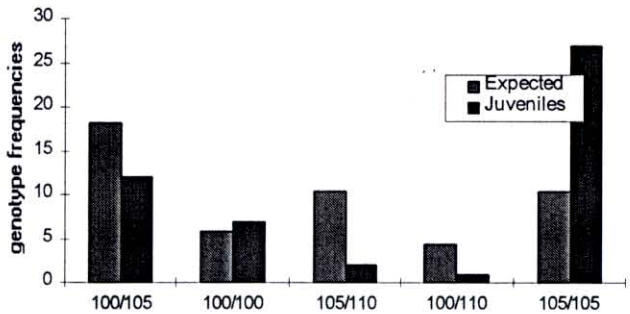
Idh-2 locus



Lap locus



Est-1 locus



EstD-2

Figure 3: Genotype frequencies observed in juveniles compared with the genotype frequencies expected from the parental allele frequencies at the ***Bonne Anse*** *population*

	Port des Barques population				**Arcachon** population				**Bonne Anse** population			
locus	genotype	Number observed	Number expected	probability of $\chi 2$	genotype	Number observed	Number expected	probability of $\chi 2$	genotype	Number observed	Number expected	probability of $\chi 2$
PGM	Homozygotes Heterozygotes	15 33	10.08 37.92	**p<0.01**	100/100 95/100 100/110 other	33 4 3 7	12.69 17.39 12.69 4.23	**p<0.001**	Homozygotes Heterozygotes	26 24	11 39	**p<0.05**
Lap	100/100 others	18 29	25.38 21.62	**p<0.05**	100/100 100/105 others	26 14 8	24.48 12.48 11.04	**NS**	95/100 100/100 others	7 38 5	10.5 15 24.5	**p<0.001**
Gpi	-	-	-	-	100/100 90/100 others	41 3 4	32.64 9.6 5.76	**p<0.05**	100/110 100/100 others	6 39 5	16 26 8	**p<0.001**
Idh-2	100/100 100/110 others	24 11 13	9.6 4.32 34.08	**p<0.001**	100/100 90/100 others	24 12 12	30.24 4.32 12	**p<0.001**	100/100 95/100 others	27 18 4	23.52 18.62 6.86	**NS**
Est-1	100/100 100/102 others	21 7 20	11.52 16.8 19.68	**p<0.005**	98/100 100/102 100/100 others	10 7 22 11	12.5 7 21 9.5	**NS**	98/100 100/100 98/98 98/102 others	18 8 16 5 3	15 12.5 4.5 4.5 13.5	**p<0.001**
Estd-2	100/105 100/100 105/105	8 17 18	21.5 17.2 4.3	**p<0.001**	100/105 105/105 others	11 28 9	23.04 17.28 3.36	**p<0.01**	100/105 100/100 105/105	12 7 27	18.13 5.88 10.29	**p<0.001**
Estd-3	100/100 others	45 2	38.07 8.93	**p<0.001**	100/100 others	44 3	32.9 14.1	**p<0.001**	-	-	-	-

Table 3: Difference in genotypic proportions between juveniles and theirs parents of the 3 populations. The expected numbers of genotypes were calculated as described in materials and methods.

3.2 Relation between heterozygosity and total weight.

Figure 4 shows no association between the number of heterozygous loci and the total individual weight in the 3 populations . The data from all 3 populations combined together show no correlation either.

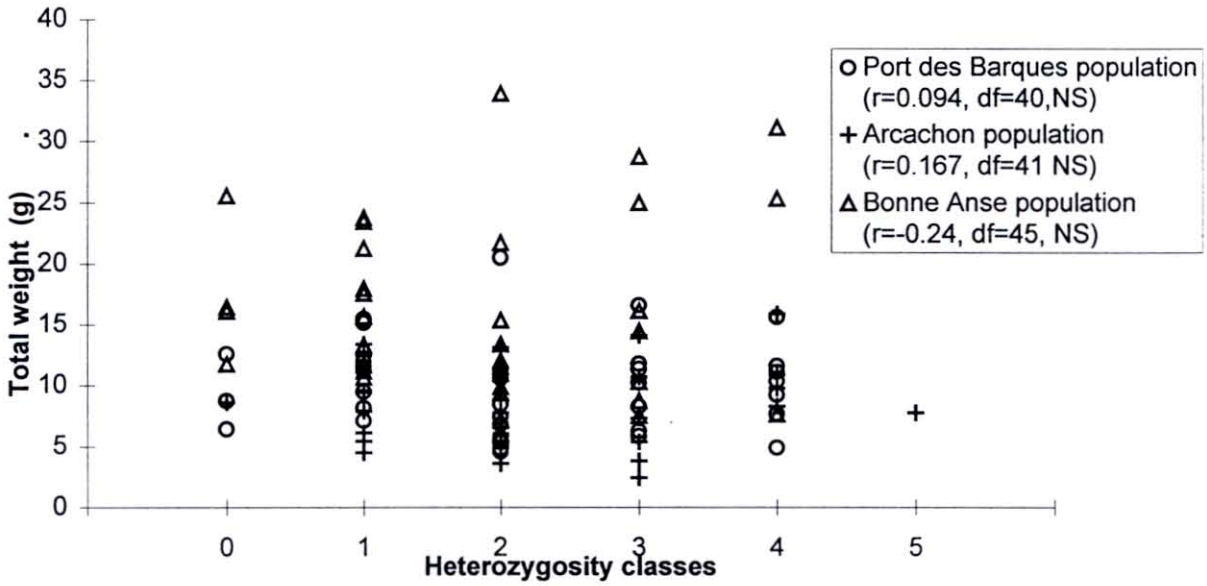
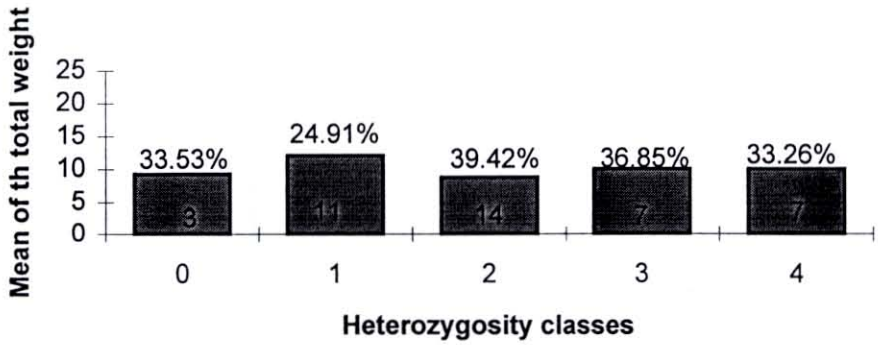
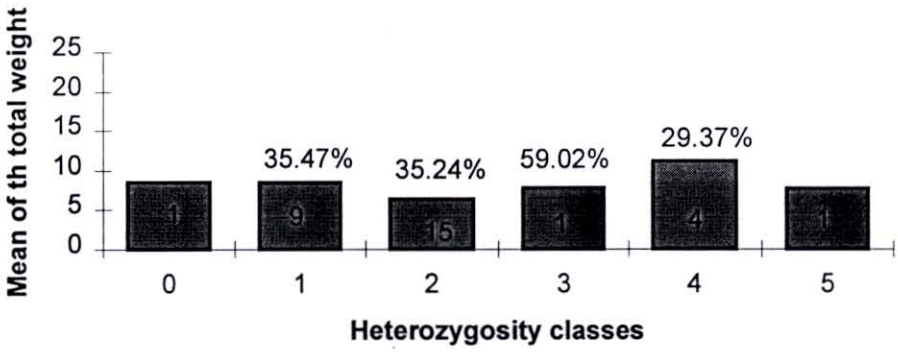


Figure 4 : Comparison between the heterozygosity classes in the three populations combined(r=-0.049, f=130, NS)

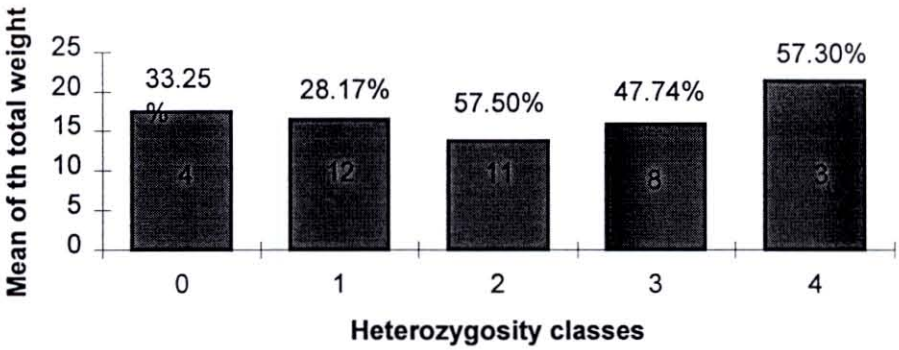
When oysters were grouped according to the number of loci at which they were heterozygous (fig 5 a,b,c), no significant differences were observed between the mean weights or between the coefficients of variation of any of the groups in any of the 3 populations. There were almost the same number of individuals in each heterozygosity class in the 3 populations.



(a) Port des Barques population (H/TW :r=0.0051,df=3 NS)



(b) Arcachon population (H/TW :r = 0.018,df=3 NS ;CV/TW :r =0.054,df=2 NS)



(c) **Bonne Anse** population (H/TW : r=0.405, df=3 NS ; CV/TW : r= 0.789, df=3 NS)

Figure 5 : Relation between the heterozygosity classes and the mean weight of the individuals within the class. *Heterozygosity classes are the number of loci heterozygous per individual. The percentages are the coefficient of variation for each heterozygosity class. The number in the columns is the number of individuals in each class. (H: Heterozygosity classes, TW : total weight, CV: coefficient of variation, r : correlation coefficient)*

The individual effect of the total weight was caculated using a T-test. The mean weights of the homozygotes and of the heterozygotes at each locus (Table 4) were not significantly different..

Population	Locus	Mean weight of Homozygotes	Mean weight of heterozygotes	Tvalue
Port des Barques	Pgm	9.009(13)	9.656(37)	0.140 (48)**NS**
	Lap	10.271(23)	8.821(27)	0.101 (48)**NS**
	Idh-2	9.847(36)	8.742(13)	0.239(47)**NS**
	Est-1	9.531(32)	9.874(16)	0.008(46)**NS**
	EstD-2	9.951(36)	10.221(7)	0.031(45)**NS**
	EstD-3	10.09(40)	10.42(2)	/
Arcachon	Pgm	6.895(35)	9.362(10)	2.283(43)*
	Lap	7.312 (26)	7.313(22)	0.011(46)**NS**
	Gpi	7.034(41)	8.859(7)	1.46(46)**NS**
	Idh-2	7.118(25)	7.549(23)	0.473(46)**NS**
	Est-1	7.243(29)	7.218(21)	0.028(48)**NS**
	EstD-2	7.437(31)	7.08(17)	0.05(46)**NS**
	EstD-3	7.249(44)	8.013(3)	0.405(45)**NS**
Bonne Anse	Pgm	16.035(24)	14.741(25)	0.786 (47)**NS**
	Lap	15.931(37)	13.84(11)	0.843(46)**NS**
	Idh-2	15.796(40)	14.614(10)	0.520(48)**NS**
	Est-1	15.364(29)	15.902(20)	0.271(47)**NS**
	EstD-2	15.011(25)	16.108(25)	0.563(46)**NS**
	EstD-3	15.09(34)	16.1(14)	0.023(46)**NS**

*Table 4 :T test between mean weight of homozygotes and heterozygotes at each locus. (* : p<0.05).*

All genotypes at each locus were compared using anova (Table 5). In the Port des barques population a significant difference was found for the Est-1 and EstD-2 loci. For Est-1, the genotypes carrying the 98 allele are significantly heavier than all others. For EstD-2, the homozygous genotype 100/100 was significantly lighter than both the other genotypes. Only 2 of 7 individuals carried both the allele 98 of Est-1 and the genotype 100/100 of EstD-2. But there were few individuals in each group. In the other two populations, there were no significant differences between genotypes.

	Port des Barques population			**Arcachon** population			**Bonne Anse** population		
Locus	Genotypes	Mean weight in g	Anova	Genotypes	Mean weigh t in g	Anova	Genotypes	Mean weight in g	Anova
PGM	homozygotes (13)	9.65	F=.283	95/100(4)	11.35		95/102(5)	13.762	
	heterozygotes (37)	9.01	p=.5974	100/110(3)	8.297		95/100(2)	10.145	
				100/100(33)	6.969		102/115(3)	14.953	
				100/102(3)	7.77	F=2.197	90/110(1)	7.62	
				110/110(2)	5.675	p=0.0867	95/100(4)	15.99	
							102/110(1)	24.95	
							95/115(1)	16.11	
							95/95(1)	23.75	
							100/100(18)	16.877	
							100/102(1)	13.36	
							100/110(5)	10.81	
							110/115(1)	23.44	
							102/102(4)	12.535	
							110/110(1)	7.17	F=1.641
							90/95(1)	31.08	p=0.1176
Lap	100/100(17)	10.57		100/105(14)	6.761		100/100(38)	15.93	
	100/110 (9)	7.758		100/100(26)	7.312	F=.597	100/105(4)	13.245	
	100/105(14)	9.964		other	8.28	p=.5547	90/100(1)	23.75	
	other homozygotes	9.471	F=1.287	heterozygotes(8			95/100(6)	12.53	F=.724
	other heterozygotes	7.22	p=0.2894	)			95/105(1)	10.28	p=.5801
Gpi	-	-	-	100/100(41)	7.034		100/100(39)	15.9	
				100/110(4)	9.12	F=1.078	90/100(4)	14.15	
				90/100(3)	8.51	p=.349	100/110(6)	12.92	F=.511
							110/110(1)	11.74	p=.6758
Idh-2	100/100 (25)	9.712		100/100(24)	7.314		100/100(27)	15.678	
	100/110(9)	8.501		95/100(3)	7.007		95/100(18)	15.677	
	110/110(7)	9.617		90/100(12)	8.563		95/110(1)	21.24	
	other homozygotes	9.374	F=.407	100/110(4)	5.793	F=758	95/95(2)	11.13	F=.378
	other heterozygotes	11.14	p=0.8025	90/110(5)	6.668	p=.5582	100/110(1)	14.61	p=.82229
Est-1	102/102(8)	8.799		98/100(10)	6.693		100/100(7)	13.37	
	100/100(21)	9.367		100/104(1)	5.37		98/98(16)	15.86	
	100/102(7)	8.381		100/102(7)	6.384		98/100(19)	15.75	
	98/98(3)	12.3		100/100(22)	7.14		100/102(1)	14.61	
	98/102(4)	15.958	F=4.723*	98/98(5)	7.182		98/102(5)	17.77	
	other heterozygotes	7.098	p=0.0016	98/104(2)	13.35		95/95(1)	17.97	F=.339
				104/104(1)	7.72		102/102(1)	9.88	p=.9126
				102/102(1)	9.37	F=1.24			
				98/102(1)	7.9	p=0.3013			
EstD-2	100/100(18)	8.324		105/105(28)	7.202		100/100(7)	16.67	
	100/105(7)	10.22	F=3.987*	100/105(11)	7.005		105/105(27)	14.45	
	105/105(18)	11.56	p=0.0275	100/100(3)	9.63	F=.591	100/105(12)	14.99	
				105/110(6)	7.217	p=.6241	10/110(1)	21.24	F=1.012
							105/110(2)	22.71	p=.4115
EstD-3	-	-	-	100/100(43)	7.326		-	-	-
				105/105(1)	3.93	F=.646			
				100/105(3)	8.013	p=.5289			

*Table 5 : Comparison by an anova of mean weight (g) of genotypes for each locus in the **3** populations. Number of individuals for each genotypes is in brakets.*

3.3 Deficit of heterozygotes

Deficits of heterozygotes were found in two populations : **Port des Barques** and **Bonne Anse** (table 6). Four loci had a deficit in the **Port des Barques** juveniles. This deficit was only present at a probability of 0.03 at Est-1 in the parents. Three loci had a deficit in the **Bonne Anse juveniles**. EstD-2 and Pgm shows a deficit in both juveniles and parents but it was larger in juveniles. There was a deficit at the Lap locus in the parents which did not exist in the juveniles.

	Port des barques		Arcachon		Bonne Anse	
	Juveniles	Parents	Juveniles	Parents	Juveniles	Parents
Pgm	.0079**	.5244	.0855	.2303	.0000**	.0225*
Lap	.1589	.1059	.2208	1	.5912	.0085**
Pgi	-	-	1	1	.3324	1
Mdh-1	-	-	-	-	-	-
Mdh-2	-	-	-	-	-	-
Idh-1	-	-	-	-	-	-
Idh-2	.0000**	.2209	.7657	1	.6724	1
Est-1	.0001**	.0309*	.0562	1	.0159*	.1163
EstD-2	.0000**	1	.3874	0.1331	.0040**	.0105*
EstD-3	1	1	.1036	1	-	-

Table 6 : Probability (Pvalue) that the genotypes were in Hardy Weinberg equilibrium in juveniles and in the parents for the 3 sites (p<0.05, ** p<0.01).*

4 Discussion

In the present study, we examined the inheritance between the parents and the offspring of 3 different wild population of *Crassostrea gigas*. Strong deviations from Mendelian expectations were observed at various loci in the 3 populations. The difference found is due either to prezygotic or to postzygotic phenomena. Even though the concentration of spermatozoa and oocytes was the same for each cross, it is known that some egg-sperm combinations in *Crassostrea gigas* often produce no spat (Lannan,1980). This may be due to either a gamete incompatibility or a difference of spawning condition. But a postzygotic phenomenon such as mortality prior to scoring can also exist. It is impossible to distinguish these two phenomena. Nevertheless, some loci did not differ from Mendelian expectations. This observation is fully compatible with selection against some genotypes at specific loci prior to scoring but not the same loci in the 3 populations.

In the present study, we evaluated the relationship between heterozygosity and growth (using total weight) in the three populations. No correlation was detected in any population. The lack of correlation between heterozygosity is not an unusual observation in a restricted mating (Beaumont, 1991). Studies on hatchery matings in scallops, oysters and mussels show a lack of any significant association between heterozygosity and growth in cohorts derived from single pair or restricted mating protocols (Beaumont, 1985 ; Foltz & Chatry,1986; Mallet,1986; Dillon,1988; Beaumont,1991). The association between heterozygosity and growth observed in natural population of bivalves is not necessarily present in hatchery populations produced from a limited number of parents (Foltz and Chatry,1986). In offspring of pair crosses in *Mytilus edulis* (Beaumont,1983), *Mulinia lateralis* (Gaffney & Scott, 1984) for example there was no association between size and heterozygosity although positive associations were observed in natural populations (Koehn et al, 1984, Gaffney &Scott, 1984)

The relationship between heterozygosity and growth depends strongly on the number of parental genomes contributing to the progeny (Gaffney & Scott, 1984). More than 40 effective progenitors are probably required before any correlation is likely to be detected in the resulting offspring (Beaumont,1991). In our study, cohorts resulted from 10 parents so it would have been unusual to find a correlation. The discrepancy between family studies and natural populations can be understood if associative overdominance is the underlying cause of the association seen in natural populations. Its basic assumption is the existence in the population of transitory linkage desquilibria between recessive deleterious genes and electrophoretic markers (Mallet *et al,* 1986). In a pair mating it is expected that allozyme can act as a marker of a very large proportion of the chromosomes which would obscure any effect of the allozymes (or linked genes) and mask the existence of the correlation (Gaffney &Scott,1986).

Zouros (1996) observed that at the Pgm locus, heterozygosity has a significant effect on growth. In our study, the Pgm locus shows a significant difference between the mean weights of homozygotes and heterozygotes but only in the Arcachon population.

In contrast with some studies of wild populations (Singh &Zouros, 1981) and another hatchery-based study involving restricted mating (Beaumont,1991) no correlation was observed here between variance in size and degree of heterozygosity.

Another common observation in marine bivalves is that the number of heterozygous indviduals is often lower than expected from Hardy-Weinberg ratios. This finding pertains to random samples from natural populations (Koehn & Gaffney,1984 ; Colgan,1987 ; Zouros, Romero-Dorey & Mallet, 1988 ; Gosling, 1989 ; Volckaert & Zouros, 1989 ; Gaffney, 1990 ; Gaffney *et al*, 1990). In our study , some loci shown a deficit of heterozygotes. When a deficit was found in the parents populations, usually it was also found in the juvenile offspring.

To sum up, our results conform with other bivalve studies using a restricted mating, ie deviations from Mendelian expectations are observed in the juvenile sample and no correlation is evident between heterozygosity and growth or heterozygote deficiency.

References

Alvarez G., Zapata C.,Amaro R.,Guerra A (1989). Multilocus heterozygosity at protein loci and fitness in the european oyster *Ostrea edulis* L. *Heredity* **63** : 359-372.

Beaumont A.R, Gosling E.M, Beveridge C.M, Budd M.D, Burnell G.M (1985). Studies on heterozygosity and size in the scallop, *Pecten maximus.In* Gibbs PE (Ed) : Proceeding of the ninetheen European Marine Biolgy Symposium" Cambridge, England : Cambridge University Press, pp 443-455.

Beaumont A.R. (1991). Genetic studies of laboratory reared mussels, *Mytilus edulis:* heterozygotes deficiency, heterozygosity and growth. *Biological Journal of the Linnean Society* **44** : 273-285.

Colgan D.J (1981). Spatial and temporal variation in the genotypic frequencies of the mussel *Barchidontes rostratus. Heredity* **46**(2) : 197-208.

Diehl W.J., Koehn R. K. (1985).Multiple-locus heterozygosity, mortality, and growth in a cohort of *Mytilus edulis. Mar Biol.* **88** : 265-271.

Dillon R.T, Manzi J.J (1988).Enzyme heterozygosity and growth rate in nursery populations of *Mercenaria mercenaria* (L.). *J. Exp. Mar. Biol. Ecol.* **116** : 79-86.

Foltz DW, Zouros E (1984). Enzyme heterozygosity in the scallop *Placopecten magellanicus* in relation to age and size. *Mar. Biol. Lett* **5**:255-263

Foltz DW and Chatry M. (1986). Genetic heterozygosity and Growth rate in Louisiana oyster (*Crassostrea virginica*).*Aquaculture* **57** : 261-269.

Fujio Y.,(1982).A correlation of heterozygosity with the growth rate in the Pacific oyster, *Crassostrea gigas.Tohoku J. Agric. Res.* :66-75.

Gaffney P.M, Scott T.M. (1984). Genetic heterozygosity and productions traits in natural and hatchery populations of bivalves. *Aquaculture* **42** : 289-302

Gaffney P.M. (1990).Enzyme Heterozygosity, Growth rate, and viability in *Mytilus edulis* : another look. *Evolution* **44** (1) : 204-210.

Gaffney P.M., Scott T.M., Koehn R.K., Diehl W.J.(1990). Interrelationships of Heterozygosity, Growth rate and Heterozygote deficiencies in the Coot Clam, *Mulinia lateralis* . *Genetics* **124** : 687-699.

Gardner J.P.A, Skibinski D.O.F. (1988). Historical and size-dependent genetic variation in hybrid mussel populations. *Heredity* **61** : 93-105.

Garton D.W, Koehn R.K, Scott T.M (1984). Multiple-locus heterozygosity and the physiological eneregetics of growth in the coot clam, *Mulinia lateralis*, from a natural population. *Genetics* **108** : 445-455.

Gosling E.M (1982). Genetic variability in hatchery-produced pacific oyters (*Crassostrea gigas).Aquaculture* **26** : 273-287.

Gosling E.M (1989). Genetic heterozygosity and growth rate in a cohort of *Mytilus edulis* from the Irish coast. *Marine Biology* **100** : 211-215

Hedgecock D., Sly F.(1990).Genetic drift and effective population sizes of hatchery-propagated stocks of the pacific oysters, *Crassostrea gigas. Aquaculture* **88** : 21-38

Koehn R.K., Gaffney P.M (1984). Genetic heterozygosity and growth rate in *Mytilus edulis. Mar. Biol.* **82** : 1-7.

Koehn R.K, Diehl W.J., Scott T.M (1988). The differential contribution by individual enzymes of glycolysis and protein catabolism to the relationship between heterozygosity and growth rate in the coot clam, *Mulinia lateralis. Genetics* **118** : 121-130.

Lannan J.E (1980).Broodstock management of *Crassostrea gigas* : I-Genetic and environnemental variation in survival in the larval rearing system. *Aquaculture* **21** : 323-336.

Lucas A., Moraga D., Marin J.(1983). Enzymatic polymorphism in two generations of *Crassostrea gigas* broodstock in a commercial hatchery. *J. Moll. Stud.* Suppt **12A** : 106-110.

Mallet A.L., Zouros E., Gartner-Kepay K.E., Freeman K.R. (1986). Genetics of growth in blue mussels: family and enzyme -heterozygosity effects. *Marine Biology* **92** : 475-482.

Moraga D., Osada M., Lucas A., Nomura T. (1989). Genetique biochimique de populations de *Crassostrea gigas* en France (cote Atlantique) et au Japon (Miyago). *Aquat. Living Resour.* **2** : 135-143.

Ozaki H., Fujio Y.(1985). Genetic differentiation in geographical populations of Pacific oyster (*Crassostrea gigas)* around Japan. *Tohoku Journal of Agriculture Research* **36**(1) :49-61.

Pogson G.H, Zouros E. (1994). Allozyme and RFLP heterozygosities as correlates of growth rate in the scallop *Placopecten magellanicus*: a test of the associative overdominance hypothesis. *Genetics* **137** : 221-231.

Raymond M. & Rousset F.(1995). GENEPOP-release 1.2- a population genetic software for exact tests and ecumenicism. *Journal of Heredity*. **86** : 248-249.

Rodhouse P.G, Gaffney P.M (1984). Effect of heterozygosity on metabolism during starvation in the American oyster *Crassostrea virginica. Marine Biology* **80**: 179-187.

Saavedra C., Zapata C., Alavarez G. (1996). Allozyme heterozygosity, founder effect and fitness traits in cultivated population of European oyster *Ostrea edulis* . *Aquaculture* **139** : 203-224.

Singh S.M. & Zouros E. (1978). Genetic variation associated with growth rate in the American oyster (*Crassostrea virginica*). *Evolution* (1978) :342-353.

Singh S.M. & Zouros E. (1981) Genetics of growth rate in oysters and its implications for aquaculture. *Can. J. Genet. Cytol.* (1981) **23** : 119-130.

Swofford D. L.& Selander R.B. (1989). Biosys-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 1.7. *In :* Swofford D.L (Ed), Illinois Natural History Survay., champaign, IL, pp. 1-43

Thiriot-Quievreux C., Pogson G.H., and Zouros E. (1992). Genetics of growth rate variation in bivalves : aneuploidy and hehetrozygosity effects in a *Crassostrea gigas* family. *Genome* **35** : 39-45.

Volckaert F., Zouros E (1989). Allozyme and physiological variation in the scallop *Placopecten magellanicus* and a general model for the effects of heterozygosity on fitness in marine molluscs. *Marine biology* **103**-51-61.

Zouros E., Singhs.M., Miles H.E.(1980). Growth rate in oysters an overdominant phenotype and its possible explanations. *Evolution* **34**(5) : 856-867.

Zouros E., Singh S., Foltz D.W, Mallet A.L, (1983). Post settlement viability in the American oyster (*Crassostrea virginica):* an overdomiant phenotype. *Genet. Res. Camb.* **41** : 259-270.

Zouros E., Romero-Dorey M., Mallet A.L.(1988). Heterozygosity and growth in marine bivalves : further data and possible explanations. *Evolution* **42**(6) : 1332-1341.

Zouros E., Thiriot-Quievreux C., Kotoulas G. (1996). The negative correaltion between somatic aneuploidy and growth in the oyster *Crassostrea gigas* and the application for the effects of induced polyploidization. *Genet. Res. Camb.* **68** : 109-116.

Task 7 : Research of chromosomal markers

The largest *C.gigas* cosmid genomic DNA library produced in the first reporting period contained 100,000 clones with an average insert length of 22 kbp. During this second reporting, a larger cosmid based genomic DNA library containing 240,000 clones has been constructed with inserts ranging in length from 6 kbp to 50 kbp. Based on comparative analysis of the genome contents of other species, this genomic DNA library is estimated to contain between two and three *C. gigas* genome equivalents. This cloning of *G. gigas* genomic DNA is now complete.

The main research emphais of the second reporting period was the development of methods to prepare suitable chromosome sspreds for *C. gigas*. Initially, gill tissue from juvenile oysters were analysed. After overnight incubation in colchicine and incubation for 30 min in 0.9% sodium citrate, the chromosomes were found to be: (i) too closely grouped together, and (ii) the chromosomes were very condensed. To optimise the methodology, assays were performed varying the time of incubation in colchicine (varied from overnigth to nine hours) and also the percentage of colchicine solution (varied from 0.005 - 0.003%). More defined and less condensed chromosome spreads were found after a nine hour incubation in 0.003% colchicine with a hypotonic incubation step of 45 min. However, the best choromosome spreads, which appear suitable for fluorescent *in situ* hybridisation analysis, were found when gill tissue from adult oysters were examined. Again the procedure used contained a nine hour incubation in 0.003% colchicine with a hypotonic incubation step of 45 min. It seems likely from these results that a greater number of mitotic cell divisions occur in adult gill tissue rather than in juveniles. These *C. gigas* chromosome spread to appear suitable for fluorescent *in situ* hybridisation analysis using DNA probes.

Two labelled cosmid clones, one containing a 24 kbp oyster genomic DNA insert and the other containing a 45 kbp insert, were labelled with the chromophore biotin-16-dUTP and used in fluorescent *in situ* hybridisation (FISH) analysis. No clear hybridisation signals were observed while low-density hybridisation signals were seen scattered among all the oyster chromosomes. These results probably reflect the fact that repetitive DNA elements found within the cosmid DNA probes are binding to homologous repetitive elements located on all the oyster chromosomes. In these experiments, the competitor DNA used to quench this type of background hybridisation was human DNA supplied within the commercial FISH kit. The FISH analysis currently in progress has replaced this competitor DNA with sonicated *C. gigas* genomic DNA. In this manner, we expect to remove the background hybridisation found among all the oyster chromosomes allowing us to detect and define chromosome specific cosmid DNA probes.

Significant difficulties or delays experienced during the first reporting period

There have been no serious delays experienced during this second reporting period. With respect to task 7, method development is due for progression to aneuploid analysis in the forthcoming reporting period. Currently, we do not have chromosome specific molecular markers defined for use in aneuploid analysis. However, we still expect to define these cosmid marekers and begin the aneuploid analysis during the upcoming third reporting period.

Annex:

METHODS

(1) Genomic DNA extraction. *Crassostrea gigas* samples were supplied by Redbank Oyster Co. New Quay, Co. Clare, Ireland. DNA was extracted using 2% CTAB (Sigma Chemical Company, ST. Louis MO. USA) and proteinase K (Boehringer Mannheim, East Sussex UK) and incubated for 1 hour at 65 C. After a series of extractions on gut, heart, muscle, mantle and gill tissue, it was found that the best concentration of intact DNA came from gill tissue. DNA was then deproteinated with phenol-chloroform isoamylalcohol (25:24:1) (BDH Laboratory Supplies, Poole, England.) and once with chloroform. The aqueous phase was adjusted to 0.3 M NaOAc (pH 5.5) and ethanol precipitated. The DNA pellet was then washed with 70% ethanol and resuspended in 200ul of sterile water and analysed on 0.8% agarose gels.

(2) Cosmid DNA preparation. Cosmids are plasmids that contain the bacteriophage lambda cos (cohesive-end site) sequences enabling the *in vitro* packaging of recombinant molecules with a minimum size of 38 kbp and a maximum size of 52 kbp. The cosmid vector used was SuperCos 1 (Stratagene Ltd, Cambridge, UK). 5 ug of vector DNA was restricted with 9U/ug of *Xba* I, followed by a second restriction with 5U/ug of *Bam* HI (Boehringer Mannheim, East Sussex United Kingdom) in standard buffer conditions at 37°C for 1 hour each. Complete digestion was analysed by electrophoresis through 0.8% agarose gels where two DNA fragments of 6.5 kbp and 1.1 kbp were visualised (i.e. indicative of complet double digestion). The cosmid vector DNA was deproteinized by phenol extraction, ethanol precipitation, and resuspended in sterile water at a concentration of 500 ng/ul.

(3) Partial digestion of oyster genomic DNA. In order to clone into the *Bam* HI site of the cosmid vector, *Crassostrea gigas* DNA was partially digested with the restriction enzyme *Sau*3AI (Boehringer Mannheim, East Sussex, UK). 30-40 ug of DNA was restricted with 0.05-0.012 U/ul of *Sau*3AI at 37°C for 15 min. Suitably digested DNA was deproteinized by phenol extraction, ethanol precipitation and resuspended in 100 ul of sterile water. To enrich for oyster genomic DNA fragments within the size of 20-40 kbp, i.e. the ideal size of insert DNA that can be cloned with the chosen cosmid vector, the oyster DNA was fractionated through a 20% sucrose (Sigma Chemical Co., St. Louis, MO, USA) gradient, centrifuged at 22,000 rpm for 15 h at 20°C. DNA fractions were analysed by electrophoresis through 0.8% agarose gels. Gradient

fractions containing DNA of 20-40 kbp in length were then dialysed using finger dialysis tubes (Schleicher & Schuell, Dassel, Germany) for 2 h. The oyster DNA was ethanol precipitated, and the pellet was washed with 70% ethanol and resuspended in sterile water at a concentration of 100 ng/ul.

(4) Cosmid vector - oyster DNA ligation reactions. The ligation reactions contained a 10 fold molar excess of vector DNA to oyster DNA molecules. The ligation reaction parameters were as follows:

1.0 ug of size fractionated *Sau* 3A1 digested oyster DNA

0.5 ug of SuperCos 1 cosmid DNA digested with *Xba* I & *Bam* HI

1.5 ul of 10x ligation buffer

1.0 ul of 10 mM rATP

Water to a final volume of 15 ul

1 ul (2U) of T4 DNA ligase (Boehringer Mannheim, East Sussex, UK) was added to the ligation reaction and incubated at 15°C overnight. A control reaction containing only the vector SuperCos 1 DNA was also performed.

(5) *in vitro* packaging reactions. The ligated DNA was then *in vitro* packaged using Gigapack II XL Packaging Extract (Stratagene Cambridge Innovation Centre, Cambridge, UK). The packaged DNA was then titered using *Escherichia coli* XL-1 Blue MR cells. (Stratagene, Cambridge Innovation Centre, Cambridge, United Kingdom) and grown on ampicillin (Sigma Chemical Co. St. Louis, MO USA) containing nutrient agar plates.

(6) Cosmid DNA minipreperations. Random cosmid colonies were chosen and cosmid DNA was extracted using the QIAprep plasmid kit (Qiagen GmbH, Hilden, Germany). The extracted DNA was then restricted with the enzyme *Eco* RI (Boehringer Mannheim, East Sussex, United Kingdom).The restricted DNA was then analysed by electrophoresis through 0.8% agarose gels to determine the size of oyster DNA inserts.

(7) Cosmid DNA labelling. DNA labelling was carried out using the nick translation technique. The DNA labels were hapten-like receptor molecules (e.g. biotin and digoxigenin) which after hybridization, are detected using the appropriate fluorescent affinity reagent. 1 ug of cosmid DNA was precipitated in ethanol and the resulting pellet was then resuspended in 66ul water, followed by addition of:

10 ul of 10x nick translation buffer

10 ul of nucleotide-mix (A,C,G)

10 ul of biotin-16-dUTP or digoxigenin-II-dUTP

4 ul of 5U/ul DNA polymerase I

1 ul of 3U/ul DNase I (diluted)

(All supplied by Bohrineger Mannheim, East Sussex, UK)

The optimal DNA fragment length for chromosomal *in situ* hybridization is between 200-500 bp. Typically, the concentration of DNase used in the nick transaltion was a 1/500 dilution of a 3U/ul stock concentration. The above reaction was incubated at 15°C for 2.5 h and then 5 ul were analysed by electrophoresis through 2% agarose gels to estimate the probe length. Once the optimal lengths were achieved, the reaction was terminated by the addition of 500 mM EDTA (BDH Laboratory Supplies, Poole, UK). The removal of unincorporated probe was carried out by gel filtration using Sephadex-50 (Pharmacia Biotech, AB, Uppsala, Sweeden).

(8) Chromosome preparation

Initially, juvenile oyster samples were collected from Redbank Oyster Co., New Quay, Co. Clare, Ireland, and incubated overnight in fresh sea water containing 0.005% colchicine (Sigma Chemical Co., St. Louis, MO, USA). Gill tissue was dissected and treated for 30 min in 0.9% sodium citrate (BDH Laboratory Supplies, Poole, UK) and fixed in a freshly prepared mixture of methanol-acetic acid (3:1) with three changes each of 20-min duration. The gill tissue was then macerated and disassociated in methanol-acetic acid (3:1). Slides were prepared by dropping the chromosomes from a height and heated on a hot plate. Chromosomes were stained using propidium iodide and visualised using a confocal microscope. The hypotonic step was varied, using 30 min, 45 min and 60 min incubation periods in order to get better metaphase spreads. A hypotonic time of 45 min was found to give the best spreads. Upon further analysis, adult oysters were noted to be undergoing more active division than the juveniles and adults were used for subsequent analysis. The percentage and time of incubation in cholchicine was varied from 6 to 9 h which yielded good chromosome spreads. Incubation in 0.003% colchicine solution was found to yield the best chromosome spreads.

(9) Fluorescence *in situ* hybridization analysis

Chromosome spreads were matured for 24 h at room temperature, pre-treated with RNase for 1 h and then prefixed in 1% formaldehyde for 2 h and dehydrated in a 70, 80, and 100% ethanol series. The chromosomes were denatured in 72°C in 70% formamide, 2x SSC and dehydrated in ice cooled ethanol series. The probe was denatured by boiling for 10 mins and added to the denatured chromosomes and *in situ* hybridization was carried out at 37°C overnight. Post-hybridization washes were performed according to the manufacturers instructions (Chromosomal *In Situ* Hybridization kit, Stratagene, La Jolla, CA, USA). Biotin labelled cosmids were detected using strepavidin FITC (fluorescin isothiocyanate) followed by biotin labelled anti-strepavidin and strepavidin FITC. Chromosomes were counterstained with propidium iodide and visualised using a confocal microscope.

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°5

IMBC
Institute of Marine Biology of Crete

Genetic Department

Reporting Period:
1st January-31st December 1997

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°5

Total cost to partner n°5 : 227.058 ECU

EC contribution : 113.529 ECU ***% of the total cost :*** 50

Duration : 60 months	***Commencement date :*** 1/1/1996 ***Completion date :*** 31/12/2000

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FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1997

Participant n°5 **Institute of Marine Biology of Crete (IMBC)**

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Scientific team **Dr Giorgos KOTOULAS**
Dr Antonis MAGOULAS
Pr. Elephterios ZOUROS
Mrs Aikaterini EKONOMAKI

Objectives :

- Participation in genetical analyses (microsatellites),
- Data exploitation and synthesis.

Actions in the project :

Task 5	Genetical analyses
Sub-task 5.2	Microsatellites in juveniles and adults, realisation of the whole Sub-task by the Partner..
Task 8	Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis.
Task 9	General organisation of the project Participation in annual scheduling meetings.

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
5.2	Lab analyses G0 / PR	Lab analyses G1 / PR	Lab analyses G1 / IS		FR
8		Data processing/ PR	Data processing/ IS	Data processing/ PR	Final synthesis/ FR
9	2 meetings with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners

PR = Periodical report
IS = Intermediary Synthesis
FR = Final Report

Research activities during the second reporting period :

Task 5 : Genetical analyses

Sub-task 5.2 : Microsatellites in juveniles and adults,

In the present study, we have utilised 4 polymorphic microsatellite loci, in order to identify parentage in 3 samples of 50 individuals (G1), obtained by 5 x 5 factorial crosses, by animals (G0) of three different regions (Arcachon, Bonne-Anse et Port des Barques). The animals of each factorial cross have been mixed together after fertilisation, so the trace of parentage has been lost. In order to attain the above target, parents and offspring have been typed for the four loci.

We have utilised the 3 loci described in the 1st Progress report of the project (Cg108, Cg49 and Cg44) and, in addition, we have typed the animals for locus Di10Cg. Partner 6 developed this marker, and the PCR conditions were optimised by partner 1. In addition we have typed 20 more offspring from Arcachon crosses, that have been utilised in the destructive physiology experiment (June 97), in order to test for correlation between performance in physiology measures, and genetic constitution.

For the parentage identification we have utilised three loci (Cg108, Cg49 and Di10Cg), since they were sufficient for an unambiguous identification of the parents of the all progeny. It should be noted that the use of only two loci, resulted in efficient identification of the parents, in about 90% of the cases.

The results of the microsatellite analyses are summarised as follows (Tables 1-4):

Arcachon cross: Parents have been identified for 50/50 individuals. One animal out of fifty analysed has been proven not to be an offspring of one of the Arcachon factorial crosses, but an offspring of a family from the Bonne Anse crosses. We characterise this animal as a "contaminant".

In this sample a high frequency of a "null allele" for locus Di10Cg has been observed in the progeny of a single female (J4). This female is homozygous for the locus Di10Cg and produced 17 offspring. The assignment of the offspring to this mother was guaranteed by the analysis based on the loci Cg108, and Cg49. After parentage identification on the basis of these two loci had been done, we observed that in 9 of its offspring she transmitted the allele, while in 8 other only the allele of the father was detected. In this case the animals appeared as homozygous for one paternal allele (which in some cases was bigger in size than the allele of the mother and in some cases smaller). Parentage identification was a necessary but not a sufficient condition

for the "null alleles" to be detected. The second condition have been the high polymorphism of the Di10Cg locus (no father had an allele identical by state, with the mother). The absence the mother's allele in some of the offspring, cannot be explained by any known mechanism of null alleles creation in microsatellites: polymorphism in the region of the primers, or competition for the Taq polymerase in the PCR tube, for alleles of very different size in heterozygous individuals.

Bonne-Anse cross: The parentage of all but one individual (49/50) has been unambiguously determined. However even in the case of this individual, which was typed for only one locus (Cg49), the parents can be identified, under the hypothesis that this animal is not a contaminant.

One of the 49 animals with unambiguous parentage determination is a contaminant produced by the Arcachon G0 population.

Port des Barques cross: Parents have been identified for 47/50 individuals. No contaminant has been found. For the three other specimens, the mother has been identified, but the father is either of two possible males. These three animals could only be typed for two loci.

Arcachon cross – June 97 experiment: Parents have been identified for 19/20 specimens. The other animal (1/20) with no resolved parentage could be typed for two loci only.

In this sample, female J4 produced 12 progeny: one could not be typed for Di10Cg. In the rest of her progeny, she transmitted 7 times a copy of her single allele, and 4 times no maternal allele has been detected. Adding up the two Archachon samples we obtain 28 progeny of the female J4. Among them, the maternal allele has been detected in 16 animals, but not in the rest 12 animals.

Table 1: Number of offspring per family, in the Arcachon crosses. F stands for Females and M for Males.

M F	J1	J2	J3	J4	J5	Total
J1	1	2	3	2	3	11
J2	1	2	3	6	8	20
J3	0	0	0	0	0	0
J4	4	3	4	1	5	17
J5	0	0	0	0	1	1
Total	6	7	10	9	17	49

Table 2: Number of offspring per family, in the Bonne-Anse crosses. F stands for Females and M for Males.

M / F	J1	J2	J3	J4	J5	Total
J1	1	6	0	0	0	7
J2	0	1	2	0	0	3
J3	0	1	0	0	0	1
J4	1	6	1	1	1	10
J5	5	17	0	4	1	27
Total	7	31	3	5	2	48

Table 3: Number of offspring per family, in the Port des Barques crosses. F stands for Females and M for Males.

M / F	J1	J2	J3	J4	J5	Total
J1	0	0	2	0	1	3
J2	0	2	12	0	2	16
J3	0	0	3	0	0	3
J4	1	0	11	0	2	14
J5	2	1	7	0	1	11
Total	3	3	35	0	6	47

Table 4: Number of offspring per family, in the Arcachon June experiment crosses. F stands for Females and M for Males.

M / F	J1	J2	J3	J4	J5	Total
J1	0	1	2	1	0	4
J2	0	0	0	1	1	2
J3	0	0	0	0	0	0
J4	1	1	4	4	2	12
J5	0	0	0	0	1	1
Total	1	2	6	6	4	19

As it can be seen from the Tables 1 to 4, the contribution of different males or females in the crosses is very heterogeneous. There are animals that contribute no progeny at all (Female J3 in Table1 and Table4, Male V4 in Table 3), while some animals contribute the majority of progeny (Female J2: 20/49, Male J5: 17/49, Female B5: 27/48, Male B2: 31/48, V3 Male: 35/47). A single family in the Bonne-Anse crosses gives 17/48 progeny (B5 x B2, Table2). For this reason (small effective population size in the beginning, becoming even smaller by an unbalanced contribution to the constitution of the next generation) a significant heterozygosity excess is observed for the loci Di10Cg and Cg108, in the samples from Archachon and Port des Barques, and for all three loci in the Bonne-Anse sample.

In the case of the locus Di10, the way of scoring the Di10Cg locus is fundamental importance for the estimation of deviations from Hardy-Weinberg equilibrium it: a) if we only consider the reading of autorads, the "null allele"-bearing animals appear as homozygotes (for a paternal allele) and we observe a low but significant deficit of heterozygotes, but b) if we infer the contribution of the mother, based on the parentage identification, then we get a high, significant excess of heterozygotes.

Results synthesis

The main objective of the microsatellite work, that is parentage identification of progeny, has been fully achieved.

1) The use of 4 highly polymorphic microsatellite loci, which allow a safe and technically easy parentage identification of the progeny in mixed or mass crosses, has been standardised. This already accomplishes the main objective of the microsatellite analysis in the project. Around 90% of parentage can be determined by using only two loci. This evidences the efficiency of these markers to this end.

2) Parentage identification is quite important for an additional reason in bivalves: crossing and rearing in these animals in order to estimate genetic parameters, can be biased by "contamination" with "immigrants", either from the wild of from other crosses, during the very early stages of development. These contaminations are relatively easy to happen, and it makes the use of microsatellites an indispensable tool in order to estimate their occurrence and extent.

3) The contribution of males and females of G0, to G1 is highly unbalanced. Some animals do not contribute at all in the next generation, and others contribute the majority of the progeny. A priory, this is unexpected, as the experiment had been designed in order to secure equal reproductive contribution of all parents. This leads to a considerable reduction of variation in the G1 generation. Although relatively important variation is still preserved, the above results should be taken into account in future experimental designs. From a Population Genetics point of view, the originally small number of males and females, coupled with their unequal contribution to the next generation, is detected by a significant excess of heterozygotes in the progeny.

4) An uncommon observation was made in the case of a female parent, which apparently is an homozygote for one locus, yet it does not transmit any allele to several of her progeny (or if it transmits it cannot be detected). The existence of a certain proportion of individuals in the G1 generation, in which the mother's allele is not detectable in the progeny, could be confounded with the segregation of a null allele in the population.

The above non detection (reflecting potentially a non transmission), could be problematic for the use of this locus for parentage identification, and could also give biased results for the estimation of Population-Genetics parameters. On the other hand we cannot exclude that this locus could be a marker of phenomena of fundamental scientific value, for a species characterised by strong segregation distortion in many loci, and loss of chromosomes.

Significant difficulties or delays experienced during the first reporting period.

There have not been any significant delays in the progress of this part of the project.

European Commission
Contract No. FAIR 95-421

"Genetic bases and variability of physiological traits involved in growth in Crassostrea gigas"
"GENEPHYS"

Individual Progress Report
1st january-31st december 1997

Partner n°6

CNRS
Centre National de la Recherche Scientifique

Laboratoire Génome et Populations, Montpellier

Reporting Period:
1st January-31st December 1997

ANNEX III
Individual Progress Report
Title page to participant n°6

FAIR PL. 95.421

" Genetical bases and variability of physiological traits involved in growth in *Crassostrea gigas*."

Individual Progress Report for the period
from **1st January to 31st December 1997**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

EC contribution : 900.000 ECU ***% of the total cost :*** 42.32

Participant n°6

Total cost to partner n°6 : 138.942 ECU

EC contribution : 69.471 ECU ***% of the total cost :*** 50

Duration : 60 months	***Commencement date :*** 1/1/1996 ***Completion date :*** 31/12/2000

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Participant n°6

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Ass. Contractor to the coordinator

Dr. François BONHOMME

FAIR PL. 95.421

Individual progress Report

for the period from
1st January to 31st December 1997

Participant n°6 **CNRS URA-1493**

Laboratoire Génome et Populations,
Université de Montpellier II
34095 Montpellier cedex 5 - FRANCE

Scientific team **Dr. François BONHOMME**
Jean Jacques VERSINI

Objectives

- Development of microsatellites markers,
- Utilisation of microsatellites markers in larvae,
- Data exploitation and synthesis.

Actions in the project :

Task 5	Genetical analyses
Sub-task 5.4	Microsatellites in larval stage Collaboration with Partner 1 for the perfection of a method for the extraction and amplification of DNA from individuals.
Task 6	Development of new microsatellite markers Realisation of the whole Task by the Partner.
Task 8	Data processing and results synthesis Data processing, participation in the results synthesis and writing of the intermediary reports and final synthesis.
Task 9	General organisation of the project Participation in annual scheduling meetings.

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
5.3	Perfectionment of the technique / PR	Perfectionment and application / PR	Lab analyses/ IS		FR
6	Research of new microsatellites markers / PR	Recherche et application / PR	Lab analyses/ IS	Lab analyses/ IS	FR
8		Data processing / PR	Data processing/ IS	Data processing/ IS	Final synthesis/ FR
9	2 meetings with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners	1 meeting with all partners

PR = Periodical report
IS = Intermediary Synthesis
FR = Final Report

Research activities during the second reporting period :

Task 5 : Genetical analyses

Sub-task 5.3 : ***Microsatellites in larval stage***

Several questions are however pending that are relevant to genetics in larval phase, that we decided to work out in the mean time on fresh material. Inheritance studies with marine bivalve molluscs often detect departures from Mendelian segregation. In controlled, pair-crosses, about half of the genetic markers, whether protein or DNA polymorphisms,show significant discrepancies between observed and expected genotypic proportions in the resulting progeny (see for instance 1, 2). Discrepancies are evident during larval development but are especially marked at the juvenile or adult stages; they arise in random-bred progenies but are striking in inbred families. The loci affected vary from cross to cross. These deviations from normal Mendelian segregation, whatever their cause, necessitate modifications of standard methods of linkage or QTL mapping. While statistical corrections are available for cases of zygotic or gametic selection, another approach is to examine segregation in very early life stages, in haploid embryos, even in gametes directly. It is possible to make haploid oyster embryos, which survive for 6-8 hrs, and we have been able (3) to produce a few such embryos.

At this stage, we have successfully amplified microsatellites from 6 hr old haploid and diploid embryos, producing enough template for 20-40 PCR reactions,.and preliminary data for one microsatellite (locus Cgi 6) suggest that segregation in both male and female parents and genotypic proportions of progeny are normal in three families. One female parent is hypothesised to carry a null allele. Further studies are needed to confirm that segregation distortion is consistently absent in early haploid or diploid stages.

For one cross we use PCR-RFLP marker on 16S DNA. This study seems to show the

existence of heteroplasmy in male gametes. An increase of the sample of the cross and the realisation of another cross should help to confirm or infirm this heteroplasmy.

Task 6 : Development of new microsatellite markers

In 1997, no new microsatellite markers have been developed. However, Partner 1 use one microsatellite locus produced in last year (locus L10) in a study about genetic of population of *Crassostrea gigas* and *Crassostrea angulata* (4). This study reinforce the hypothesis of the asiatic origin of Portugese Oyster (*Crassostrea angulata*). The results confirm a precedent study on PCR-RFLP markers(5).

Significant difficulties or delays experienced during the second reporting period :

After having produced a set of microsatellites loci as reported in last year report, we intended to work on the larval offsprings of the 5x5 diallele cross at various stages. However, the attempts we made to amplify the larvae that had been fixed and put aside were unsuccessful, and revealed that PCR amplifiability was decreasing after a few month of storage. We have thus to work again on the storage conditions of the larvae, or to be able to proceed rapidly to the analysis of the future crosses.

Reference

(1) McGoldrick, D.J. and D. Hedgecock, 1997. Fixation, segregation and linkage of allozyme loci in inbred families of the Pacific oyster *Crassostrea gigas* (Thunberg): implication for the causes of inbreeding depression. Genetics 146: 321-334

(2) Bierne, N., Launey, S., Naciri-Graven, Y., et Bonhomme, F., 1998. Early effects of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. Genetics, Mar. 98 (in the press).

D. Hedgecock, D. Brun, and F. Bonhomme. Genetic mapping in oysters : problems with non Mendelain segregation and complete interference. Communication to the Plant & Animal Genome VI meeting (San Diego, January 18-22, 1998)

Huvet A., 1997. Différenciation génétique de deux huîtres creuses *Crassostrea gigas* et *Crassostrea angulata* : Apport des marqueurs microsatellites. Rapport de DEA. Faculté des Sciences et Techniques de Tours. 25p

Boudry P., Heurtebise S., Collet, B., Cornette F., and Gérard A., 1998. Genetic differenciation between Portugese [*Crassostrea angulata* (Lamark)] and Pacific [*Crassostrea gigas* (Thunberg)] oyster populations, as reveled by RFLP analysis of PCR amplified mitochondrial DNA segments. *Journal of Experimental Marine Biology and Ecology*.