Commission of the European Communities

Contract No. FAIR 95-421

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

THIRD PROGRESS REPORT
1st January-31st December 1998

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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Abstract

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" 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 1998**

Type of contract : Shared-cost research project

Total cost : 2.126.745 ECU

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

Duration : 60 months

Commencement date : 1/1/1996

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Abstract

1. Objectives

The EU 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, using 2 types of markers (allozymes and microsatellites), the growth differential of physiological functions is associated with a genetic basis, and whether this is 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

This study uses experimental populations of the Pacific cupped oyster, *Crassostrea gigas,* to examine genetic and physiological mechanisms behind growth patterns in this species. To this end, experimental progenies were produced using oysters from different populations to provide a large genetic base for investigation. The crosses were made principally in 1996, the first year of this five year project. The resulting generation, G1, has been studied in depth for characters of interest and the possible relationships between genetics and physiology. Work on the project has involved the development of new techniques in different fields of biology in order to examine these traits on the animals. Study of G1 occupied much of the second and third years of the project and has provided a large amount of data, much pertaining to a common group of individuals on which several different experiments were conducted. We are now in a position of synthesis for the data gained for the G1 generation from work done by the 6 different partners and 9 laboratories involved in the project. The G1

generation was also used to produce a G2 generation, which consists of different progenies which will be used for different investigations. The breeding of a second experimental generation from the first will enable us to gain an idea of inheritance of physiological parameters important for productivity in the species. One set of genitors were selected from G1 animals according to physiological performance and a second were chosen for levels of somatic aneuploidy (missing chromosomes). The G2 was successfully produced in 1998 and studies on this generation will commence in 1999.

3. State of progress

The 5-year project has now reached the end of its third year. In the first year, efforts were mainly dedicated to the production of the first experimental generation G1 and development of techniques. Year two saw an emphasis on the application of methods for genetic and physiological study on this biological material. Partners initiated a co-ordinated study on a group of common animals. In year three, work on the first experimental population has continued and practical work is reaching an end. Results gathered on the physiological performances and levels of aneuploidy in different animals were used to select parents for the breeding of the second experimental generation in which the levels of these characters will be examined.

Production and management of the G2 generation

This task was performed in 1998 using animals scored for the stability and efficiency of physiological performance in 1997-1998 and populations displaying different levels of somatic aneuploidy. The first part of the G2 generation is presently being raised under controlled conditions in preparation for growth and physiological study in the coming two reporting periods. It consists of progenies of crosses between combinations of parents of high and low performance. 4 progenies have already been selected for study and individual size measurements begun. G2 animals for the study of inheritance of aneuploidy were bred using parents from populations of contrasting aneuploidy level. 4 progenies available for study issue from crosses between and within groups of high and low aneuploidy.

Recording of growth performances

The recording of growth performances on the G1 generation was completed this year with the collection of final data from a group of animals grown at Palavas-Etang de Thau. The results indicate differences in growth patterns between this 'semi-natural' environment and that of Bouin where controlled experiments on growth and competition were conducted in the second reporting period. The influence of initial size on growth and final size was re-emphasised but it is clear that in the natural environment, smaller animals may achieve greater size increases relative to their initial size than large animals. Uniform conditions in contrast tend to favour uniform growth between size classes. The difference in results also suggests that genotype x environment interaction occurred, as the genetic material used was similar and that these studies should be followed up by an in depth experiment on individual growth in the natural environment.

ANNEX I

Abstract

Physiological Analysis

According to the last annual reports two main experiments were performed in 1997 and 1998: temporal stability of physiological traits and characterisation of physiological performances of animals from the G1 generation. At present all the experiments have been finished to schedule. Data from each participant are completely treated.

The development of new continuous or sequential measurement systems for respiration and filtration, have allowed animal activity to be quantified over periods of 24 hours.

The physiological performances of laboratory raised animals were shown to be stable over time (based on 5 experiments over the course of a production cycle of 8 months). This result shows that in production conditions where food is not a limiting factor for growth, animals which perform the best at one period of the year also perform the best during other seasons.

Parents for the 2nd generation were ranked according to an index incorporating the activity and intensity of physiological functions concerning the acquisition and use of energy. The animals with the highest performance have greater absorption and lesser oxygen consumption than those with the lowest performance.

For the first time in this study a linear relationship between oxygen consumption and animal dry weight was observed ($p<0.01$).

The existence of high variation in absorption could be explained by the existence of two sub-groups which react differently to changes in food availability. If this hypothesis were correct, it would show that acclimation to low food supply can rapidly induce effects on oxygen consumption, while regulation of absorption would be faster or slower depending on the animal.

A similar distinction between G1 animals was found in the digestive enzyme activity study. The division of these animals into two sub-groups based on absorption efficiency data appears to be supported by genetic study. It remains to be shown however, whether the two sub-groups determined in the enzymatic activity study correspond to the two sub-groups distinguished by the non-destructive measurements on physiological functions.

Activities of both leucine aminopepdidase and cathepsin D were higher in the remaining tissues of slower-growers, confirming that the faster whole-body protein synthesis in slower-growers stems from fundamental metabolic differences in non-digestive tissues, and were not directly related to feeding rate.

Development has been completed of a novel assay for cytosolic calcium-dependent proteases will tell us whether differences in whole-body protein turnover derive solely from extracellular lysosomal activity, or whether they also stem from associated differences in basal intracellular metabolism.

Genetic Analysis

The allozyme and microsatellite analysis has been completed on G1. The methods both show that there is a an imbalance in parental contribution in the progeny. Microsatellite analysis performed on larvae and juveniles from one set of G1 controlled crosses show that parental contribution is unbalanced from the earliest stages of life, that it changes over time (a feature already exposed in the allozyme study) and that gametic competition occurs during fertilisation. Results of the microsatellite study of G1 adults emphasised that only a limited number of parents

used in the crosses made for G1 may be represented in the progeny and therefore that genetic variation is less than the potential suggested by the parent used in a crossing design. Parental effects on growth in the growth experiment in Bouin will be analysed in the coming reporting period.

Allozyme techniques were applied to the study of heterozygotes in G1 this year. Heterozygote deficiency did not appear to differ consistently with age class, i.e. despite indications from microsatellites that overall variability is reduced, this does not appear to be manifested as a reduction in heterozygotes with increasing age of the experimental population. Deficit of heterozygotes did not appear to be linked with growth slope either but did appear at greater levels for some loci in the progeny of some intra-population crosses more than others.

Aneuploidy study has advanced with the completion of banding maps for the identification of missing chromosomes in aneuploid cells. Aneuploidy itself has been examined in animals of different size from contrasting populations once again showing that there is a negative relationship between aneuploidy and size and that there is a population (genetic) effect. In the coming reporting period, the untangling of size and genetic effects will be examined using samples of the same size from a series of full-sib families treated for chromosome study this year. The inheritance of aneuploidy will be examined in G2 families issued from crosses made this year between the populations with different levels of this trait.

4. Achievements

The experimental studies on the G1 have now been completed.
This year these have yielded:

- Results from the growth experiment at Thau indicating that the influence of initial size is environment dependant and that small oysters can achieve higher specific growth than large ones.

- The confirmation of stability of physiological traits (oxygen consumption and filtration) over a period of 8 months during which the same individuals were measured repeatedly. Different individuals showed different performances and degrees of stability but overall statistics showed animals to be stable.

- The demonstration that faster whole-body protein synthesis in slower-growers stems from fundamental metabolic differences in non-digestive tissues, and is not directly related to feeding rate.

- The support for enzymatic results distinguishing different sub-groups of an experimental sample (digestive enzymes activities, digestive enzyme Michaelis constants) by genetic differences.

- The application of allozymes and microsatellites showing that parental contribution is unbalanced and changes over time.

- The scoring of aneuploidy in populations of different origin and animals of different size confirming the negative correlation of the trait with size and providing further evidence for a genetic basis.

- The creation of G2 progenies for study of physiological traits and aneuploidy.

- The analysis data by each partner individually.

In the context of this study of genetics and physiology of *Crassostrea gigas,* new techniques have also been developed. This year these include; completion of G and R banding maps for the identification of missing chromosomes in the karyotype, a chromosome specific probe for use in FISH analysis and the assay for cytosolic calcium-dependent proteases for application to protein degradation study.

5. Future Actions

Genetic and physiological analysis on the G2 generation which will be completed in 1999:

- Study of individual growth performances

- Physiological performances (non-destructive study of filtration and oxygen consumption)

- Protein turn-over and proteolitic activity

- Digestive enzyme activities

- Inheritance of aneuploidy

Work will continue to integrate the G1 data from the different experiments completed in the preceding reporting periods. This should provide important insight into the genetics and physiology of growth in *C.gigas.*

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Scientific Synthesis

1. Introduction

The 5 year project has now reached the end of its third year. With the production of experimental populations and development of techniques in the first two years, in this third we have been able to :

- Apply techniques in physiology (continuous monitoring, stress proteins and proteolitic enzymes) and genetics (allozymes, microsatellites) to the study of the populations raised in experimental conditions,
- Examine results on the first experimental generation (G1) based on growth, physiology and genetic studies,
- Make selective crosses for a second experimental population in which we will investigate the inheritance of the traits studied in the first experimental generation.
- Make advances in techniques for the use of chromosome markers and enzyme activity studies.

2. Results

Tasks 1 and 2 Production and management of the G1 and G2 generations

The first experimental generation (G1), based on 3 intra population 5X5 crosses was produced in 1996. The second experimental generation (G2) was produced this summer (1998) in the hatchery at La Tremblade. The crosses for G2 offspring were made with parents from the G1 generation, selected for their physiological performances (see task 4). Eighteen crosses were made in all over two dates. A third cross is planned using G1 individuals as parents in early 1999. In addition to these crosses, a small group of 'genephys' G1 oysters were crossed with individuals from a totally separate population showing low aneuploidy (Scotland) in order to study the possibility of inheritance of this phenomenon (see task 5).

Task 3 Recording of the growth performances

Experiments done in the first reporting period investigated the role of size in subsequent growth in oysters from a very young age. Experiments followed on from these to look at how such relationships changed as oysters grew older and how competition affected growth.
This year, the growth recording on the G1 generation was completed with the final measurements being made at Palavas (Etang de Thau) on an experimental set from two of the original 5X5 crosses. These oysters were studied in the traditional

local farming system of cementing the animals to ropes which are then suspended in the water on frames. As in the growth experiment conducted at Bouin in the second reporting period, the oysters were initially sorted into size classes. The farming system prevented all but initial and final measurements of size but based on these two measurements we can see that there is a linear relationship when initial weights are plotted against final weights. This implies that differences between individuals that exist at an early stage will be maintained in adult life. At Thau in contrast to Bouin, neither population or weight class had an influence on total gain in weight but it was noted that the gain in weight was strongly inversely related to the initial weight, i.e. smaller animals grew more during the experimental time and unlike the experiment completed last year at Bouin, no strong relation between initial size and growth rate was detected.

The results gathered at Thau differ in some respects from those obtained at Bouin. This could indicate some genotype x environment interaction, a result of the longer duration of the experimental time (over 3 months), or a direct influence of the specific culture system used. Conditions at Thau were not uniform between size classes because small oysters were grouped on ropes with small oysters and large with large, whereas at Bouin, competition levels were finely controlled.

Growth studies will be made on the G2 population starting by the recording of individual size from the juvenile stage. These results will later be compared with physiological parameters of the same animals.

Task 4 Physiological analysis

According to the last annual reports two main experiments were performed in 1977 and 1998:

1) temporal stability of physiological traits,

2) characterisation of physiological performances of animals from the G1 generation.

At present all the experiments are achieved. Data from each participant are completely treated.

Temporal stability of physiological traits

Physiological functions have typically been measured over periods varying from 1-6 hours. Data would then be extrapolated, using energy budget equations, to longer periods such as a day, a month or even a year, without taking into account the activity of these animals over longer periods.

The development of new continuous or sequential measurement systems (described in previous annual reports) have allowed animal activity to be quantified over periods of 24 hours. Using these techniques in the present experiments, respiratory activity was found to be variable. It was lower in some experiments (respiratory time activity varying from 65-75%), notably during the experiment of April 1997 (2nd annual report, Bougrier et al., 1998) than in others (around 85%).

These differences could be due to differences in the conditions between the laboratories where the animals were raised, variable temperature and unlimited food. For the moment it is difficult to interpret data on filtration activities (Filtration time activity, FTA) because these are the first data recorded. However, the FTA values recorded in this study, which are of the order of 70%, are comparable with those in the literature obtained by measuring the valve activity.

The physiological performances of laboratory raised animals were shown to be stable over time (based on 5 experiments over the course of a production cycle of 8 months). A Friedman rank test is significant for all the characters studied (Oxygen consumption, filtration, absorption and scope for growth). This result shows that in production conditions where food is not a limiting factor for growth, animals which perform the best at one period of the year also perform the best during other seasons.

The animals of this sub-task were used as genitors for the 2nd generation G2 (task 2). The animals were ranked according to an index incorporating the activity and intensity of physiological functions concerning the acquisition and use of energy. This index is based on the mean of 5 absorption values (OAR, representing energy acquisition) and 5 oxygen consumption values (OCR, representing the energy costs). The animals with the highest performance (greatest OAR/OCR ratios) have greater absorption and lesser oxygen consumption than those with the lowest performance (lowest OAR/OCR ratios).

Characterisation of physiological performances

In 1997 a group of G1 animals, acclimatised for 15 days to 15°C and low food levels ($2mgl^{-1}$) were each studied for all the following characters

1. Non destructive measurement of physiological functions
2. Measurement of digestive enzyme activity
3. Estimation of protein turn over
4. Determination of proteolitic activity

Most physiological functions depend on animal size. The relationships of oxygen consumption and filtration with dry weight are best described with allometric models. This signifies that small animals consume proportionally more food and oxygen than large animals. An important finding of the present study is a linear relationship between oxygen consumption and dry weight ($p<0.01$). This is the first time that oxygen consumption of small animals has been observed to be proportionally equal as that of larger animals. This difference can be explained by 1) the fact that this study uses animals of the same age class, while results previously published in the literature were obtained on animals from several age classes. 2) The animals had experienced a stress. From the start of their lives until the acclimation period of the present experiment the animals had an unlimiting food supply, then during the acclimation, they were supplied a diet characterised by very low food availability.

This stress appears to have had an effect on absorption. In effect, the relationship between this physiological function and dry weight is highly variable. The existence of high variation could be explained, even without statistical proof,

by the existence of two sub-groups which react differently to changes in feeding. Absorption by animals in the first sub-group would be linearly related to dry weight whilst in the 2nd sub-group this relationship would be allometric. The linear relationship in the first group could be induced by acclimation to low food availability. If this hypothesis were correct, it would show that acclimation to low food supply can rapidly induce effects on oxygen consumption, while regulation of absorption would be faster or slower depending on the animal.

A similar distinction between G1 animals was found in the digestive enzyme activity study. The results show that animals in one subgroup would be better adapted to low seston load than those in a second subgroup. The animals in the first sub-group would have a better absorption efficiency under non-limiting conditions, but a lower growth potential due to lower absorption at low seston load. Conversely, the animals of the second sub-group would have a lower performance under non-limiting conditions because of their low absorption efficiency. The study therefore seems to show two types of animal,

1. Animals with high growth due to high absorption and a high absorption efficiency in non-limiting raising conditions, but which display a low growth performances in conditions where food is limiting due to a low absorption efficiency.
2. Animals with low growth and low absorption whatever the food availability conditions, but which nevertheless have better growth potential in limiting conditions than the animals with high growth.

The division of these animals into two sub-groups based on absorption efficiency data appears to be supported by genetic study. It remains to be shown however, if the two sub-groups determined in the enzymatic activity study correspond to the two sub-groups
distinguished by the non-destructive measurements on physiological functions.

The data obtained today give promising results as to our knowledge, it is the first time in molluscs that it has been possible to show agreement between independent data from a physiological approach (measurement of absorption efficiency), an enzymatic approach (digestive enzymes activities, digestive enzyme Michaelis constants), and a genetic approach (different genetic patterns of amylase gene).
A model can summarise a part of this convergence, as absorption efficiency is depending on enzymatic activities (E) and Ingestion rate (ING), with a saturation process when ingestion rate exceeds the Michaelis constant Km. These catalytic constants are related to genetic differences.

In the present programme, slower rates of protein synthesis in faster-growing oysters indicate that a greater proportion of that synthesis must have been effecting net protein deposition than in slower-growing oysters, so that the intensity with which proteins were being replaced and renewed (= protein turnover) was greater in those slower-growers. This suggests, as in past studies, that slower growth in the first GI generation of this GENEPHYS programme resulted at least in part from the high energetic costs of protein turnover, together with faster rates of nitrogen excretion.

Activities of both leucine aminopepdidase and cathepsin D were higher in the remaining tissues of slower-growers, confirming that the faster whole-body protein synthesis in slower-growers stems from fundamental metabolic differences in non-digestive tissues, and was not directly related to feeding rate. These findings also establish that differences in whole-body protein turnover and growth efficiency stem from extracellular proteolytic activity, for both leucine aminopepdidase and cathepsin D are located mainly in lysosomes.

Development of a novel assay for cytosolic calcium-dependent proteases has been completed. This will tell us whether differences in whole-body protein turnover derive solely from extracellular lysosomal activity, or whether they also stem from associated differences in basal intracellular metabolism.
Whilst developing our assay for calcium-dependent proteases, we have found calcium-dependent activity in the adductor muscle of *C. gigas*, which degraded proteins from this same tissue within a pH range of 7.4 to 8.4 and optimally at pH 7.8. This pH optimum is higher than those reported for other molluscs.
Optimal elution conditions also differ from those in other molluscs. Calcium-dependent proteases from *C. gigas* elute at a salt concentration above 0.25 M and optimally between 0.3 M and 0.35 M. This differs markedly from the 0.2 M salt concentration at which calcium-dependent proteases elute in octopus.

Tasks 5 and 6 Genetic analysis and Development of Microsatellite markers

Genetic markers

This year, the microsatellite markers developed in the first two reporting periods were put to use in the study of both a developing larval population and adult G1s from the integrated inter-partner experiment started in June 97.

The analyses made on the adult G1 oysters from the integrated 'June 97' study and from the Bouin growth experiment show an imbalance of parental contribution similar to results found comparing GO and G1 with microsatellites in the previous reporting period. Parental contribution is unbalanced at the adult stage of the progeny and this reduces genetic variation. In the adult study, different physiological groups from the 'June 97' experiment appear to have distinct parentage. The study on larvae done this year sheds more light on parental contributions.

The larval population studied were produced from crosses of parents with known alleles at one microsatellite locus. This enabled parental analysis to be made on samples of the larval offspring. The contributions of different parents to the progeny were seen to change over time as the young oysters developed and evidence for gametic competition was shown by differences between the progeny resulting from a cross made by mixing all gametes and a cross made with the same parents but with each parental combination crossed separately before mixing. Size differences were not apparent between different families or between the offspring of different parents at the stages studied up to 90 days old but differences in contribution started from the earliest stage observed: 6 days old.

Allozyme study was also used on animals from the G1 generation. There was a strong deviation from Mendelian expectations and, as found with

microsatellites, parental contributions were seen to be unbalanced. Further work was done on heterozygosity this year. Heterozygote deficiency was detected but not with consistent patterns between populations or between juveniles and adults. Heterozygotes were occasionally found to be smaller than homozygotes and an unusual negative relationship was found between growth and heterozygosity in two out of the three original intra-population crosses. This contrasts with the results from last year where no correlations were found.

Aneuploidy

A sample population of G1 raised at Bouin were used to make a comparison of aneuploidy in oysters of different size. 30 animals were divided by size into small, where 22% aneuploidy was found, medium (17% aneuploidy) and large (6% aneuploidy). This pattern follows that found last year where somatic aneuploidy was inversely correlated with growth rate in oysters from the original intrapopulation crosses. For comparison, a control population from outside the genephys populations was studied, 15 small and 15 large oysters originating from Argenton (Scottish broodstocks) showed 16% and 5% aneuploidy respectively. Once again, growth showed a negative correlation with aneuploidy.

Transmission of aneuploidy will be examined between the G1 and G2 generations. Selected oysters from the genephys G1 generation were treated for chromosome examination and used as parents in a cross with non-genephys animals from the Argenton (Scottish broodstocks) in La Tremblade in July 1998 to provide G2 material. The progeny of 4 crosses between and within these groups will be examined in 1999.

As an extension to the investigation of the relationship between aneuploidy and growth, a group of full-sib families were examined in order to compare within and between family variation and to see if family effect was distinct from size effect. 330 animals were treated for chromosome study (La Tremblade, July 1998, collaboration Partner 3 - Partner 1).

The development of chromosome marker techniques has involved two major methods, firstly the characterisation of the bands visible on mitotic metaphase chromosomes and secondly, in situ hybridisation with labelled molecular probes (task 7).

This year for the first technique, worked on by Partner 3, the G and R banding has been successfully completed meaning that we are now able to identify each of the different chromosomes of the *C. gigas* karyotype.

Task 7 The search for Chromosome Markers

In situ hybridisation techniques have produced a functional probe for use on metaphase cells this year. Following the development of a DNA genomic library, 28 probes were tested for *in situ* hybridisation on metaphase cells. Out of these 28 probes there was the one that gave good results (i.e. single locus hybridisation, allowing the distinction of one chromosome pair) and 4 others which could produce results with improved protocols.

Task 8 Data analysis and results

In the third reporting period there has been further analysis on growth physiology and genetics data obtained last year. The experiments made on the G1 generation were completed this year, and their data gathered. Their integrated analysis will take place in the following reporting periods. The 'June 97' experiment offers the possibility for the comparison of physiological and genetics data on the same animals.

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 Galway in November 1998 allowed each partner to be informed of all the different tasks of the project and to discuss future actions (see Annex 4).

3. Discussion-Conclusion

The third year has concluded the practical work done on the progeny of the first crosses made in the study (G1). A considerable amount of data has been gathered and analysed. Further integration of this data analysis will take place over the following reporting periods to present an overall picture. Present progress has given some new and interesting results and tools for future research.

Growth recording has shown differences between the development of oysters at the different sites (Bouin and Thau) indicating possible genotype x environment effects and differences between the natural environment and the controlled experimental environment. Future experiments should incorporate individual monitoring methods into experiments in the natural environment in order to examine these differences more closely. The new results continue to emphasize the importance of size differences in early life for subsequent development.

Physiological studies now indicate an overall stability for animals in 5 repeated monitoring studies made over one year. Differences in physiological efficiency were shown with some individuals displaying high energy intake accompanied by low metabolic cost. Animals with extreme values were chosen as parents for the G2 so as to look at the effect of parental performance on the progeny. From the experiment with common genetic material, it can already be seen that larger oysters distinguish themselves from smaller oysters by a lower protein turnover and lower proteolytic activity. Study of digestive enzymes implies that the efficiency of the oysters metabolism may be dependant on the food available and that two groups are present in the study population with respect to ability to adapt to high and low food availability.

The genetic part of the study has shown a basic support for microsatellite results by those obtained with allozymes. Both methods show that juvenile and adult populations differ in genetic structure and that parental contributions are unbalanced.

The data from microsatellite study on larvae shows that gametic competition takes place and that unequal contribution arises from the earliest stages.

Aneuploidy has once more been found to be inversely related to the size of the animals and this year was seen to be lower in a population from outside the French production zones. Work on chromosome markers has progressed substantially yielding a FISH probe and completed data on G and R band chromosome identification tools

In all, the project has achieved the objectives set for this third reporting period, and has made significant progress in the study of growth, physiology and genetic analysis of the experimental material. This lays a firm foundation for the continuation of studies on the next 'Genephys' oyster generation and on the examination of inheritance of the traits of interest.

Future Actions

Analyses to be performed on the G2 generation in 1999:

- Individual growth performances
- Continuous monitoring of physiological performances
- Digestive enzyme activities
- Proteolytic and protein turnover studies
- Microsatellite analysis on later larval stages
- Measurement of levels of aneuploidy

Further analyses and in depth integration of the work by the different partners will be made on the G1 data in 1999. 1998 has produced the new generation G2 for these studies as well as considerable extra information about G1. In the coming year the experiments on G2 will offer the opportunity of intergeneration comparisons of performance and inheritance for insight into the heritability of growth characteristics in oysters.

FAIR PL. 95.421

Methodology and research tasks

Consolidated Progress Report **for the period**
from 1st January to 31st December 1998

Task 1 : Production and management of the G1 generation
Participant : 1 (IFREMER La Tremblade, Bouin, Palavas)
Duration : 36 months
Current status : Completed
Total estimated Man-months : 12 man-months
N° of man-months already devoted to the task : 17.5 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 third reporting period:

This part of the programme has been successfully completed

Sub-task 1.1 : Crosses between G0 parents
Participants : 1
Duration : 27 months (initial estimation:15 months)
Current status : Completed
Total estimated Man-months : 9.5 man-months
N° of man-months devoted already to the task : 9.5 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 third reporting period:

The bulk of this part of the programme was completed in the previous reporting periods. There was a reduced number of offspring due to mortalities (see 2nd year report) but enough oysters survived to continue the planned experiments. The latest crosses for G1, which were made in 1998, were used for the microsatellites on larvae (task 5.3) study.

Sub-task 1.2 : Breeding of G1 pseudo-cohorts
Participants : 1 (IFREMER Palavas)
Duration : 27 months
Current status : Completed
Total estimated Man-months : 2.5 man-months
N° of man-months already devoted to the task : 8 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 third reporting period:

This part of the programme was completed in the previous reporting periods.Young oysters were divided between Thau and Bouin for subsequent growth experiments (task 3) as described in the 2nd year report for safety reasons and to conduct experiments in the highly controlled conditions at Bouin as well as in the natural environment at Thau.

Task 2 : Establishment and management of the G2 generation
Participant : 1 (IFREMER La Tremblade, Bouin)
Duration : 42 months
Current status : 24 months to completion
Total estimated Man-months : 15 man-months
N° of man-months already devoted to the task : 8 man-months

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 third reporting period:

G2s have been established consisting of 11 and 7 full sib families respectively for two sets of crosses made in June and August based on physiological performances and 12 full sib families were established for the study of aneuploidy.

Sub-task 2.1 :	Selection of G1 Parents and crosses
Participants :	1 (IFREMER La Tremblade, L'Houmeau) et 3
Duration :	15 months
Current status :	Completed (theoretically)
Total estimated Man-months :	11 man-months
N° of man-months already devoted to the task :	5 man-months

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 third reporting period:

It was decided to make crosses based on the physiological performances of the G1s. The ranking of the 30 G1 oysters from the Bonne Anse intra-population cross studied for their physiological performances in 5 successive continuous monitoring experiments (task 4.1), was available in June. The chosen parameter was the ratio of food absorption to oxygen consumption rates (expressed in terms of energy) rather than scope for growth. Therefore, the best oysters were those which assimilated the most energy and consumed the least. Two crosses were attempted in June and August 1998 with these animals. Thermal induction only caused spawning in some individuals and due to a lack of females amongst these some animals were stripped. The crosses have resulted in 11 and 7 full-sib families respectively. 19 of the original parents survive and a third set of crosses is planned for 1999.

G2 for aneuploidy transmission was produced from crosses between 'Genephys' oysters and oysters from another population displaying a lower level of aneuploidy. Crosses were made within and between these groups in July 1998 at

La Tremblade. Three "2 x 2" crosses, between oysters from populations presenting contrasting levels of aneuploidy, were performed in 1998 (P1a, P3). Parents were sampled to estimate their individual aneuploidy rates. The 12 full-sib progenies (≈ 3000 oysters/family except one family with less offspring) are growing in La Tremblade.

Sub-task 2.2 : Breeding of G2 families
Participants : 1 (IFREMER La Tremblade, Bouin)
Duration : 30 months
Current status : 24 months to completion
Total estimated Man-months : 4 man-months
N° of man-months already devoted to the task : 3 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 third reporting period:

4 progenies from the first cross have so far been selected. For each of the families chosen for study in the physiology/growth part of G2, 100 animals were individually weighed and placed in divided trays so that growth could be monitored without the influence of competition.

Task 3 : Recording of the growth performances
Participant : 1 (IFREMER La Tremblade, Bouin, Palavas)
Duration : 57 months
Current status : 21 months to completion
Total estimated Man-months : 10 man-months
N° of man-months already devoted to the task : 11 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 third reporting period:

Experiments done in the first reporting period investigated the role of size in subsequent growth in oysters from a very young age. Experiments followed on from these to look at how such relationships changed as oysters grew older and how competition affected growth (experiment at Bouin).
This year, the growth recording on the G1 generation was completed with the final measurements being made at Palavas (Etang de Thau) on an experimental set from two of the original 5X5 crosses. These oysters were studied in the traditional local farming system of cementing the animals to ropes which are then suspended in the water on frames. As in the growth experiment conducted at Bouin in the second reporting period, the oysters were initially sorted into size classes. The farming system prevented all but initial and final measurements. Based on these two measurements we can see that:

- There is a linear relationship when initial weights are plotted against final weights. This implies that differences between individuals that exist at an early stage will be maintained in adult life.

- At Thau in contrast to Bouin however, neither population or weight class had an influence on total gain in weight.

- At Thau it was noted that the gain in weight was strongly inversely related to the initial weight, i.e. smaller animals grew more during the experimental time.

- Unlike the experiment completed last year at Bouin, no strong relation between initial size and growth rate was detected.

Growth studies will now be started on the G2 population starting by the following of individual size from the juvenile stage. These results will later be compared with physiological parameters of the same animals.

Task 4 : Physiological analyses
Participant : 1 et 2
Duration : 57 months
Current status : 21 months to completion
Total estimated Man-months : 45 man-months
N° of man-months already devoted to the task : 46.6 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 third reporting period:

The last physiology experiment with the generation G1 were completed in 1998. Treatment of the data was conducted and all results are now available. The total integrated treatment (of the data on all characters from all three partners) cannot be done before 1999, after the assembly and distribution of a common file containing all the data from the June 1997 experiment.

Sub-task 4.1 : Analysis of the temporal stability of the physiological traits
Participants : 1 (IFREMER L'Houmeau)
Duration : 36 months
Current status : Completed
Total estimated Man-months : 6 man-months
N° of man-months already devoted to the task : 12.6 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 third reporting period:

In 1996, it was decided to do 5 experiments to increase the precision of a test of possible temporal stability of physiological performances.
In 1997, 3 of the 5 experiments had been done in September, October and December. Only the preliminary results from the September and October experiments were presented in the second annual report.
In 1998, the last two experiments were conducted in February and April. The analysis of the five experiments is now finished and shows the stability of the physiological characters through time. In addition, the animals studied in these experiments were used as genitors for the breeding of the G2 generation (sub-task 2.2). An index allowed us to sort the animals from the lowest to the highest performances according to their physiological characteristics. The animals with the highest performances were characterised by higher energy aquistition (absorption) and lower energy consumption than those with lower performances.

Sub-task 4.2 : Non destructive measurements of physiological traits
Participants : 1 (IFREMER L'Houmeau)
Duration : 42 months
Current status : 18 months to completion
Total estimated Man-months : 8 man-months
N° of man-months already devoted to the task : 9 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 third reporting period:

In 1997, physiological performances were studied on animals from the three intrapopulation crosses made in 1996. At the end of this experiment (June), these animals were sent to other partners in the program for physiological and genetic analysis. Only preliminary results were presented in the second year report.
In 1998, the treatment of data was completed. Physiological traits measured showed no significant differences between animals issued from the different intra population crosses, even though growth was significantly different between these groups.
The results of this study show for the first time, and in contrast to data in the literature, that oxygen consumption is linearly related to dry weight of the animal.
The relationship between absorption and dry weight is more complex to interpret. It appears that two subgroups can be distinguished, characterised by their ability to adapt quickly to low seston load. One presents an allometric relationship and the other a linear one.

Sub-task 4.3 : Proteolytic activity and protein turn-over measurements
Participants : 2
Duration : 48 months (initial estimation: 42 months)
Current status : 18 months to completion
Total estimated Man-months : 27 man-months
N° of man-months already devoted to the task : 16 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 third reporting period:

Protein turn-over and net ammonium losses were recorded on 40 out of the 68 oysters (20 large + 20 small). These parameters were found to be negatively allometrically related to dry weight. Moreover, they are significantly higher in individuals which are heterozygote at the DAP locus (recorded by P4) than in homozygotes. This is not the case for lysosomal proteins. Additionally, activities in digestive gland, gill and remaining tissues have been recorded in fast and slow growing oysters from the Bouin experiment (July 1997).
Faster growing oysters showed slower rates of protein synthesis indicating that a greater proportion of the protein synthesised must have resulted in deposition than in slower growing individuals. The greater protein turnover in the slower growers indicates they have poorer efficiency. A study of enzymes in the different tissues showed that leucine aminopepdidase and cathepsin D had higher activities in the remaining (rather than digestive, gill or adductor muscle) tissues of slower growers indicating that it is not digestive related activity which causes then differences in protein turnover and in growth observed.
A new assay has been developed for CANP (Calcium-dependant Protease).

Sub-task 4.4 : Digestive enzyme activity measurement
Participants : 1 (IFREMER Brest)
Duration : 48 months (initial estimation: 42 months)
Current status : 18 months to completion
Total estimated Man-months : 6 man-months
N° of man-months already devoted to the task : 9 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 third reporting period:

In 1997, we modelled absorption efficiency using a Michaelis Menten-like expression combining relative ingestion rate, and oyster specific digestive enzyme activities (amylase and laminarinase). More than 50% of the variance was explained by this model and two different physiological constants were obtained characterising two subgroups named KM1 and KM2 within the 60 samples analysed from the June experiment. One subgroup had a high absorption efficiency at low ingestion rate, the other had the same high absorption efficiency value only when ingestion rate was high.
In conclusion of the last report we planned to determine the significance of these physiological constants.

1998 activities : were focused on these 3 objectives :

- Km of amylase and laminarinase of available samples among the 60 from June were determined.
- PCR were performed on genomic DNA extracts of these samples (provided by Dr Kotoulas, partner 5 from Greece) using adapted primers.
- A set of G1 oysters placed at Thau was also sampled in spring 1998 to evaluate performances and digestive activities in this special trophic situation and specific activities are under analysis.
- Sampling of G2 at young larval stages, previously planned for this stage of the project was delayed, because it seemed a priority to first understand the earlier results obtained on broodstock at a deeper level.

The results demonstrated :
1- That Km of the two enzymatic activities varied on a large scale in the samples analysed, suggesting a mixture of amylase proteins from different genes or alleles. But no significant difference was observed between enzymatic Km of the two subgroups.
2- Significant correlations between amylase Km, absorption efficiency and relative growth rate were shown. Separation of these data between the two subgroups KM1 and KM2 demonstrated that the above correlations were mainly observed in the KM2 population.
3-The genetic approach using amylase cDNA primers and PCR techniques demonstrated size polymorphism in the genomic amplified region of an amylase,, suggesting the existing of at least two genes and explaining results of point 1.
4- Four categories of samples were characterised demonstrating different genetic patterns.

A significant difference was observed between the amylase Km value and the genetic amylase pattern, demonstrating that this parameter was genetically dependant.
5- The genetic pattern for amylase overlapped between subgroups KM1 and KM2, but seemed different in two minor parts of the pattern.
6- A significant relationship between relative growth performance and the genetic pattern was observed for three of the genetic groups and a trend was observed for all four of the groups, supporting the results of point 2.
7- Enzymatic activities of subgroup KM2 were significantly correlated with absorption efficiency but not in subgroup KM1.

The two subgroups differed mainly in the relationships between digestive enzyme parameters, physiological parameters and genetic pattern and these differences were clear in all but one of these parameters.
All these results combining physiological measurements, enzymatic analyses, and a genetic approach are in accordance with the proposed model. However, these results come from a limited number of individuals (maximum 60) and the three primary objectives should be improved working with the other Genephys partners.
The existence of two physiological subgroups needs to be shown to be a repeatable result. Amylase polymorphism has to be analysed using different approaches (molecular and genetic). Corresponding enzymatic properties have to be estimated, in relation to physiological parameters. These steps will be undertaken in 1999 using different available samples from G0, G1 (Thau 98 and June 97 experiments), and the G2 generation, if available.

Task 5 : Genetic analyses
Participant : 1,3,4,5 et 6
Duration : 57 months
Current status : 21 months to completion
Total estimated Man-months : 100 man-months
N° of man-months already devoted to the task : 94.15 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 correlations 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 third reporting period:

The allozyme and microsatellite analysis has been continued for parentage analysis of G1 with some analysis and synthesis remaining for microsatellite analysis on adults and larvae. Allozyme techniques were applied to the study of heterozygotes in G1 this year. With the collation of this data the work with allozymes and microsatellites will be complete.

Aneuploidy study has advanced with the completion of banding maps for the identification of missing chromosomes in aneuploid cells. Aneuploidy itself has been examined in animals of different size from contrasting populations once again showing that there is a negative relationship between aneuploidy and size The inheritance of aneuploidy will be examined in G2 families issued from crosses made this year between the populations with different levels of this trait.

Sub-task 5.1 :	Allozymes	
Participants :	4	
Duration :	36 months	
Current status :	Completed	
Total estimated Man-months :		14 man-months
N° of man-months already devoted to the task :		23 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 third reporting period :

All genetic analyses using allozymes have been completed and correlated with growth parameters where these are available.

Sub-task 5.2 : Microsatellites on adults and juveniles
Participants : 5
Duration : 48 months (Initial estimation: 36 months)
Current status : 12 months to completion (including extension)
Total estimated Man-months : 38 man-months
N° of man-months already devoted to the task : 30.85 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)). Wewill ensure the availibility of new mocrosatellite loci by creating a new DNA bank and screening it(*Task 6, Partner 6*).

Progress in the third reporting period :

3 polymorphic microsatellite loci were used to identify parentage of
a) 68 (G1) offspring that had been utilised in the non-destructive physiology experiment (June 97) (task 4.2).
b) 181 individuals (G1) from the Bouin-Growth experiment (task 3).
Unbalanced contribution of parents was found in the G1 progeny. Differences in parentage were observed between the different physiological groups (see task 4.4) and a spontaneous triploid was detected.

Sub-task 5.3 : Microsatellites on larvae
Participants : 6 et 1 (Partner 6 leader of this Sub-task)
Duration : 54 months (Initial estimation 42 months
Current status : 18 months to completion (including extension)
Total estimated Man-months : 20 man-months
N° of man-months already devoted to the task : 15.3 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 :

Microsatellites markers were used to do a parental analysis at different age stages on the progenies of two 5 x 5 crosses in order to evaluate the parental contribution and influence of gametic competition. Genitors were chosen to have known genotypes for a microsatellite marker. They were chosen from a random sample of 100 oysters from the French Atlantic coast. Biopsies were performed on these animals and DNA extracted by the Phenol-Chloroform method. From the 100, 5 males and 5 females were chosen with different microsatellite sequences at the L10 locus in order to have parents with identifiable offspring. The parents were chosen to be heterozygote at L10 and to have no shared alleles in order to ease the task of parental analysis in the next generation and avoid ambiguity caused by null alleles.

The possible effect of the gametic competition on the parental contribution to the following generation (selection or preferential mating) was evaluated by the comparison the of 2 types of crosses : with gametic competition (mixed fertilisation en masse) and without gametic competition (separate fertilisations). Individuals were sampled at 6 days, 18 days and 90 days after fertilisation, and DNA was extracted (Chelex method) and PCR amplified for the microsatellite analysis of parental contributions. Sampled individuals were also measured using a Nikon profile projector and at 90 days the samples were also weighed.

The analysis of different larval and post larval stages allowed the study of evolution of relative parental contribution through time, and the detection of any differential growth or mortality between progenies.

Progress in the third reporting period :

In this period we put microsatellites developed in previous reporting periods into use in a parental analysis of a developing progeny.

The parents were shown to have differing contributions to the progeny and these contributions changed as the young developed. There was a significant difference in male contributions but not in female contributions at certain stages. Certain individuals in particular had large contributions to the progeny though all alleles from all genitors were represented in the population.

Evidence for gametic competition was shown by differences between the allelic representation in the progenies of the two crosses. However only male contributions were significantly different between the mixed (with gametic competition) and separate (without gametic competition) crosses. The male contributions were more irregular in the mixed cross than in the separate cross, indicating the occurrence of spermatic competition. Female contributions were very heterogeneous but not different between crosses.

Of the 25 male x female combinations giving families, there was a significant difference in representation of the families. Imbalance between families was

attributed to individual gametic contributions rather than male x female interaction which was not significant at any stage.

It is clear that imbalance starts at an early stage. From the present results, segregation distortion of the pairs of alleles from each parent occurs in all but one parent. No cross analysed at any stage, shows any significant difference in size (or weight) between either the 25 families or between the offspring of the individual males or females. Nevertheless, significant differences of size are observed at D6 between the mixed cross which produced larger individuals, and the separate cross (p=0.0001). This could result from selection against inferior individuals from as early as the zygotic stage.

Work will continue into the fourth reporting period to examine the parental contributions and family composition in the later stages of the mixed (with gametic competition) cross progeny.

Problems encountered in the third reporting period :

The 'microsatellites on larvae' analysis was planned to be done on sets of G1 larvae sampled in a similar way over time but starting at a much earlier stage, at only 24 hours old. This plan presented problems of DNA conservation in frozen 24-hour-old embryos so a later time interval was chosen and the new parents and crosses made to a) generate replacement material and b) have parent of known and traceable microsatellite genotypes.

Sub-task 5.4 : Karyotype analysis
Participants : 3 and 1 (Partner 3 is leader of this Sub-task)
Duration : 57 months
Current status : 21 months to completion
Total estimated Man-months : 31 man-months
N° of man-months already devoted to the task : 25 man-months

Objectives :(see above)

Materials and methods:

Ovocytes 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 third reporting period :

- Study of aneuploidy in a control population originating from Argenton (Scottish broodstocks). 15 small and 15 large animals were studied.

- Study of aneuploidy in 36 tagged animals of three size classes originating from the Bouin population where growth and allozymes were simultaneously investigated.

- Study of aneuploidy transmission. 12 parents were processed for aneuploidy study by a destructive method (La Tremblade, July 1998). Chromosome slides of 8 parents were scored.

- Study of aneuploidy in a group of full-sib families to assess the relation aneuploidy-growth within and between families. 330 animals were treated for chromosome study (La Tremblade, July 1998, collaboration with Partner 1).

- Identification of chromosomes of C. gigas. "G"-bands patterns were obtained for all chromosome pairs. "R" banding gave complementary results.

Task 6 : Development of new microsatellite markers
Participant : 6
Duration : 24 months (initial estimation: 48 months)
Current status : Completed
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.

Materials and Methods:

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 third reporting period :

This task has been completed and the microsatellites produced are now in application on the genetic problems in tasks 5.2 an 5.3 which represent the continuing work.

Task 7 : Search for chromosome markers
Participant : 4
Duration : 54 months
Current status : 18 months to completion
Total estimated Man-months : 24 man-months
N° of man-months already devoted to the task : 16 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 5.4) 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 third reporting period :

One set of probes has been successfully visualised on metaphase spreads and three others are being optimised. When optimised, these will be used on aneuploid spreads.

Task 8 : Data processing and results synthesis
Participant : 1, 2, 3, 4, 5 and 6.
Duration : 54 months
Current status : 18 months to completion
Total estimated Man-months : 30 man-months
N° of man-months already devoted to the task : 21.2 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 third reporting period :

Data processing has continued to be completed by each partner individually. The 'June 97' inter-partner study is the first experiment where the same animals were used in diverse studies. The data has only just been all received and so combined analysis and discussion will made during the next reporting period.

Task 9 : General organisation of the project
Participant : 1, 2, 3, 4, 5 and 6.
Duration : 60 months
Current status : 24 months to completion
Total estimated Man-months : 15 man-months
N° of man-months already devoted to the task : 11.3 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.

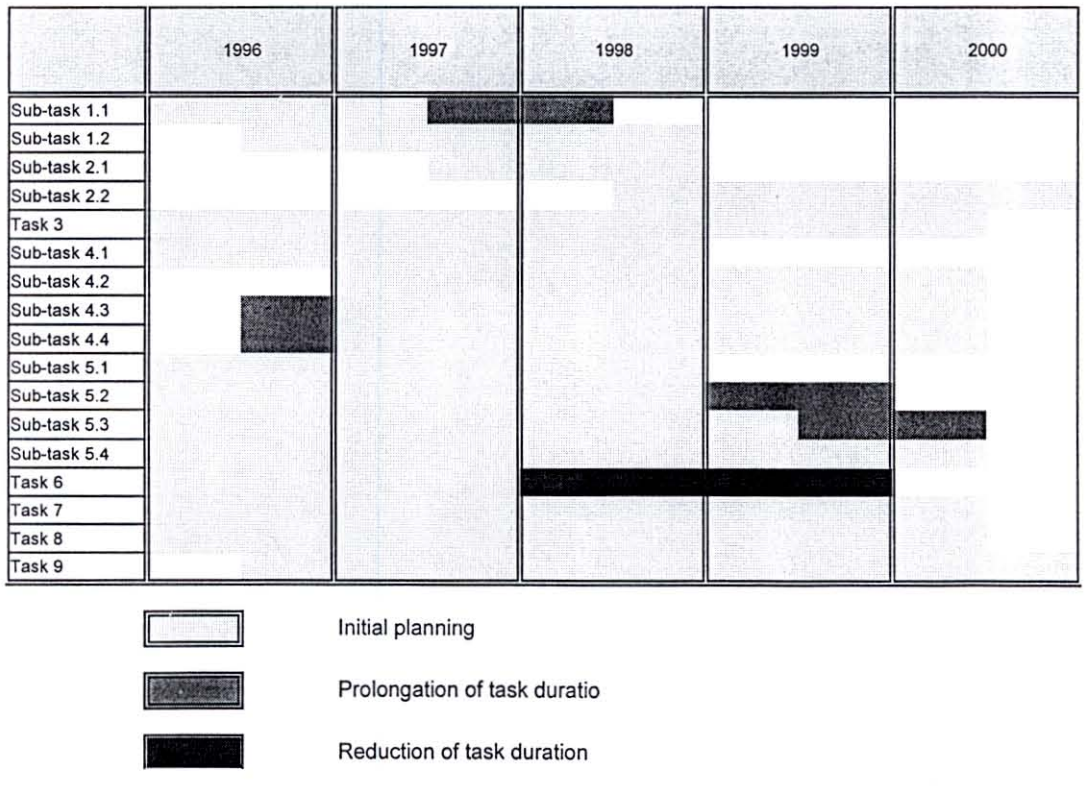
Progress in the third reporting period :

Co-ordination for organisation of finances, reports and joint tasks continues primarily though the use of email from La Tremblade. Several bi- and tri- partate meetings were organised during the year for the planning of experiments etc. The annual meeting with all partners took place in Galway on the 2nd- 3rd November 1998, allowing the exchange of information on the various tasks in the programme. The minutes of this meeting are included as an annex (4) to the present report.

Milestone

The following tables sum up the progress status of each sub-task with regard to the scheduled project :

Operation	in preparation	started	running	finished
Sub-task 1.1				finished
Sub-task 1.2				finished
Sub-task 2.1				finished
Sub-task 2.2		as scheduled	as scheduled	
Task 3		as scheduled	as scheduled	
Sub-task 4.1				finished
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				finished
Sub-task 5.2			prolonged until the end of 1999	finished
Sub-task 5.3		as scheduled	prolonged until the year 2000	
Sub-task 5.4		as scheduled	as scheduled	
Task 6				finished (in advance)
Task 7		as scheduled	as scheduled	
Task 8		as scheduled	as scheduled	
Task 9		as scheduled	as scheduled	



ANNEX II
Consolidated Progress Report
Methodology and research tasks

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			G1 generation, 1st and 2nd sets	
Sub-task 2.1			Parents selected on growth performances, crosses made	
Sub-task 2.2			Young G2 oysters in hatchery	*G2 generation*
Task 3			Growth of G1s	*Growth of G2s*
Sub-task 4.1			Temporal stability of performances measured on G1	
Sub-task 4.2	Non-destructive measurement protocols of physiological traits		Non-destructive measurements of physiological traits on G1	*Non-destructive measurements of physiological traits on G2*
Sub-task 4.3	Assay for calcium dependant proteases		Protein turnover and Proteolic activity on G1	*Protein turnover and Proteolic activity on G2*
Sub-task 4.4			Digestive enzyme activity on G1	*Digestive enzyme activity on G2*
Sub-task 5.1			Allozymes on GO and G1 adults and juveniles	
Sub-task 5.2			Microsatellites on G1 adults	
Sub-task 5.3	Microsatellites tested on embryos		Microsatellites on larvae	*Microsatellites on larvae*
Sub-task 5.4	Banding techniques, Completed banding maps		Aneuploidy on G1,	*Aneuploidy in G2*
Task 6	5 new microsatellite markers			
Task 7	Cosmid genomic DNA library, 1 chromosome-specific probe	*Chromosome Identification probes*		*Chromosome Identification*
Task 8	Treatment of G1 physiology data by separate partners	*Creation of a integrated G1 data file for the 'June 97'*		
Task 9	Progress reports 1,2 and 3	*4th Progress report, Final report*		

Dissemination

Posters and Oral presentation

1998

Collet B, Boudry P, Thebault A, Heurtebise S, Morand B, Héral M and Gérard A. (1998). Relationship between pre- and post-settlement growth in the Pacific oyster Crassostrea gigas (Thunberg). Talk at the Aquaculture Europe '98 Conference, Bordeaux, France, October 7-10.

Boudry P, Collet B, Kotoulas G, Magoulas A, Hervouet V, Bohomme F, Gérard A. The use of microsatellite markers for parentage analysis in the Pacific cupped oyster, Crassostrea gigas (Thunberg). Talk at the ICES Annual Science Conference – 86th Statutory Meeting, Cascais, Portugal, September 16-19.

Collet B. (1998). The genetic bases of the physiological traits involved in growth in Crassostrea gigas. Presentation of the EU program at the European Conference in Marine Biology and Fisheries, Lisbon, May 22-28.

1997

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

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

Publications

1999

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

Leitao A, Boudry P, Labat JP, Thiriot-Quiévreux C. Comparative karyological study of cupped oyster species. Malacologia, **in press**.

1998

Baud JP., Palvadeau H., Nourry, M., Haure J., Penisson, C., and Boudry ¨P. (1998) Bases génétiques et variabilité des caractères physiologiques impliqués dqns la croissance de *Crassostrea gigas.* Regional report SMIDAP N°97 05266, September 1998.

Boudry P, Collet B, Kotoulas G, Magoulas A, Hervouet V, Bohomme F, Gerard A. The use of microsatellite markers for parentage analysis in the Pacific cupped oyster, Crassostrea gigas (Thunberg). ICES CM 1998/K:7.

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

Collet B (1998) Bases génétiques des charactères physiologiques impliqués dans la croissance chez l'huitre creuse *Crassostrea gigas* Phd Thesis, Institut National Agronomique Paris-Grignon

Hervouet, V. (1998) Utilisation d'un marquer microsatellite pour l'étude des contributions parentales chez l'huître creuse *Crassostrea gigas.* DEA Thesis, Université des Sciences et Techniques de Tours.

1997

Hawkins AJS. and Day AJ. (1997) How protein metabolism can 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 1998

Partner n°1

IFREMER

Institut Français de Recherche pour l'Exploitation de la Mer

Laboratoire Génétique, Aquaculture et Pathologie (GAP)

La Tremblade

Laboratoire Conchylicole de pays de Loire

Bouin

Centre de Recherche en Ecologie Marine et Aquaculture

L'Houmeau

Laboratoire Méditerranéen de Recherche en Aquaculture

Palavas

Laboratoire de Physiologie des Mollusques

Brest

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual Progress Report
Participant n° 1

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 1998

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

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Coordinator : Dr André GERARD
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Coordination genetic aspect :
Dr Pierre BOUDRY
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Coordination physiological aspect :
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(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

ANNEX III
Individual Progress Report
Participant n° 1

FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1998

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

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Dr. Pierre BOUDRY,
Dr. Sylvie LAPEGUE,
Emmanuel GOYARD,
Serge HEURTEBISE,
Christophe LEDU,
Pascal PHELIPOT,
Bertrand COLLET (PhD. Student).

Laboratoire Conchylicole de pays de Loire
Dr. Jean-Pierre BAUD
Max NOURRY,
Jean-Louis MARTIN.

Centre de Recherche en Ecologie Marine et Aquaculture (Unité mixte IFREMER/CNRS L'Houmeau) (Partner : P1b)
Dr. Maurice HERAL,
Dr Serge BOUGRIER,
Herve CHARTOIS,
Francoise MORNET,
Pierre GAREN,
Florence CORNETTE (CDD),
Helen MCCOMBIE (CDD).

Laboratoire Méditerranéen de Recherche en Aquaculture (IFREMER Palavas) (Partner : P1c)
Dr Dominique BUESTEL,
Jean-Michel CHABIRAND,
Jocelyne OHEIX.

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

ANNEX III
Individual Progress Report
Participant n° 1

Objectives :

Obtaining and managing the biological material
Recording growth of the biological material
Participation in genetical analysis
Mesurement 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 crossbreds, a)parental sorting on physiological criteria by the P1b Partner, b)parents crossbreds by the P1a Partner.
Sub-task 2.2	Breeding G2 families realisation of the whole Sub-task by the P1a Partner.
Task 3	Recording of the growth performances 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 mesurements of the physiological functions realisation of the whole sub-task by the P1b partner.
Sub-task 4.4	Mesurement of the 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)Karyotypes 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. Coordination by the Partner 1 of this Task.
Task 9	General organisation of the project Coordination by the Partner 1 of this Task..

ANNEX III
Individual Progress Report
Participant n° 1

Planned Research Activities :

Task	Year 1	Year 2	Year 3	Year 4	Year 5
1.1	Crossbreeding/ PR				FR
1.2	G1 breeding/ PR	Breeding G1 / PR	Breeding G1 / PR		FR
2.1		Parents assortment /PR	Crossbreeding/ PR		FR
2.2			Breeding G2 / PR	Breeding G2 / PR	Breeding G2 / FR
3	Data acquisition G1/ PR	Data acquisition G1/ PR	Data acquisition G1 et G2 / PR	Data acquisition G2/ PR	Data acquisition G2 / FR
4.1	Physiological measurement / PR	Physiological measurement G1/ PR	Physiological measurement G1 / PR		FR
4.2		Physiological measurement G1/ PR	Physiological measurement G1 / PR	Physiological measurement G2/ PR	Physiological measurement G2 / FR
4.4		Physiological measurement G1/ PR	Physiological measurement G1 / PR	Physiological measurement G2/ PR	Physiological measurement G2 / FR
5.3	Perfectionment of the technique / PR	Perfectionment and application / PR	Lab analyses / PR	Lab analyses / PR	FR
5.4	Ovocytes analyses G0 / PR		PR	Study of aneuploidy transmission / PR	Study of aneuploidy transmission / FR
8	Data processing and statistical analyses / PR	Data processing and statistical analyses / PR	Data processing and statistical analyses / PR	Data processing and statistical analyses / PR	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
FR = Final Report

Research activities during the third reporting period :

Task 2 Establishment and management of the G2 generation

Sub-task 2.1 Selection of G1 Parents and crosses

1. Introduction

Three sets of crosses were performed at La Tremblade to produce oysters for the G2 generation. Two were made using oysters chosen by their physiological performances (recorded in task 4.1), the third was done in collaboration with Partner 3 to examine the inheritance of aneuploidy and is described in the partner 3 individual progress report.

2. Materials and Methods, Results

A group of G1 oysters from the Bonne Anse intrapopulation cross (1st progress report) were studied for their physiological performances, based on the 5 successive sets of measurements over a year (Task 4.1). An index was conceived (mean assimilation rate/mean oxygen consumption) that incorporated the level of physiological performance with the level of stability over the year studied and thus enabled the oysters with stable high or low physiological performance to be identified. This data was available in June 1998. Despite the fact that some of these oysters spawned spontaneously in their tank the 14th of May, 17 (3 females and 14 males) out of the 27 oysters spawned following a thermal induction on the 23rd of June. 20 full sib families were finally made by stripping of 2 contrasting females (numbers 1 and 26). Stripping was necessary in order to obtain enough female gametes. Out of these 20 crosses, 11 full sib families, called G2.1 were settled on cultch and are growing in la Tremblade (Table 1).

Females Performance Rank: 1 low-27 high (old reference number)		Males Performance Rank: 1 low-27 high (old reference number)	Offspring status (10/98) Mean Weight	Number
1 (09B6)	X	2 (14B9)	0.1186	1156
1 (09B6)	X	8 (09C7)	0.4152	376
1 (09B6)	X	24 (14H1)	0.1414	904
1 (09B6)	X	27 (15G9)	0.0586	1761
26 (06B9)	X	2 (14B9)	0.2092	602
26 (06B9)	X	3 (13F3)	0.3005	276
26 (06B9)	X	5 (15G4)	0.1657	281
26 (06B9)	X	21 (09E7)	1.0648	103
26 (06B9)	X	22 (13B7)	>3mm 0.0308 >7mm 0.1888	1909 763
26 (06B9)	X	24 (14H1)	0.5639	291
26 (06B9)	X	25 (06C9)	0.5575	116

Table 1 Crosses made 23rd June 1998. G1 crosses producing G2.1 full-sib families

The 26th of August, a second cross was attempted using the 19 remaining oysters. Seven male oysters spawned but no females. These 7 males were crossed with a single common female (which had not been studied for physiology). 7 full sib families, called G2.2 were settled on cultch and are growing in la Tremblade (Table 2). The 19 parental oysters are still alive and a 3rd set of crosses is planned for 1999 (parental status is summarised in annex 4 of this report: minutes of the annual meeting).

Females: I sole female:F Not previously scored for physiology		Males Performance Rank: 1 low-27 high (old reference number)
F	X	2 (14B9)
F	X	3 (13F3)
F	X	9 (13G9)
F	X	10 (06B2)
F	X	18 (15A1)
F	X	23 (14B7)
F	X	24 (14H1)

Table 2 Crosses made 26th August 1998. G1 crosses producing G2.1 full-sib families

Problems encountered in the third reporting period

As indicated above, when the crosses were made, there was a problem of sex ratio which limited the crosses possible and meant that some oysters had to be stripped.

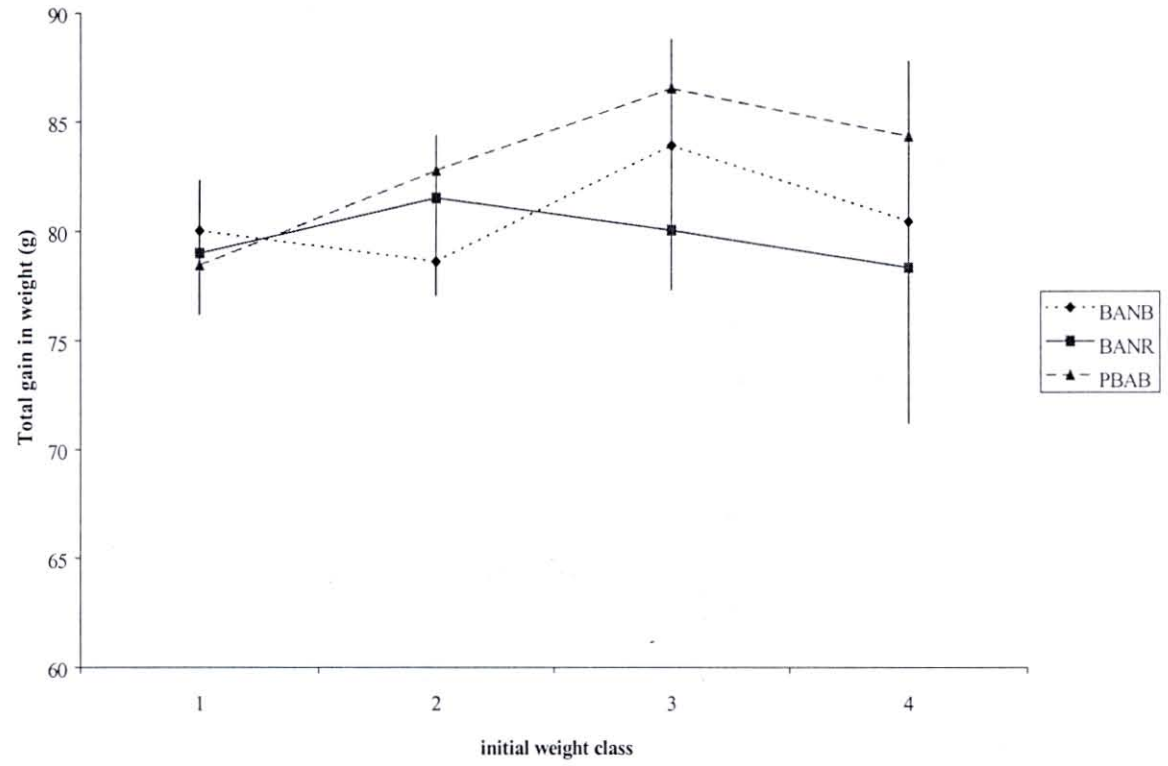
Sub-task 2.2 Breeding and raising of the G2 generation

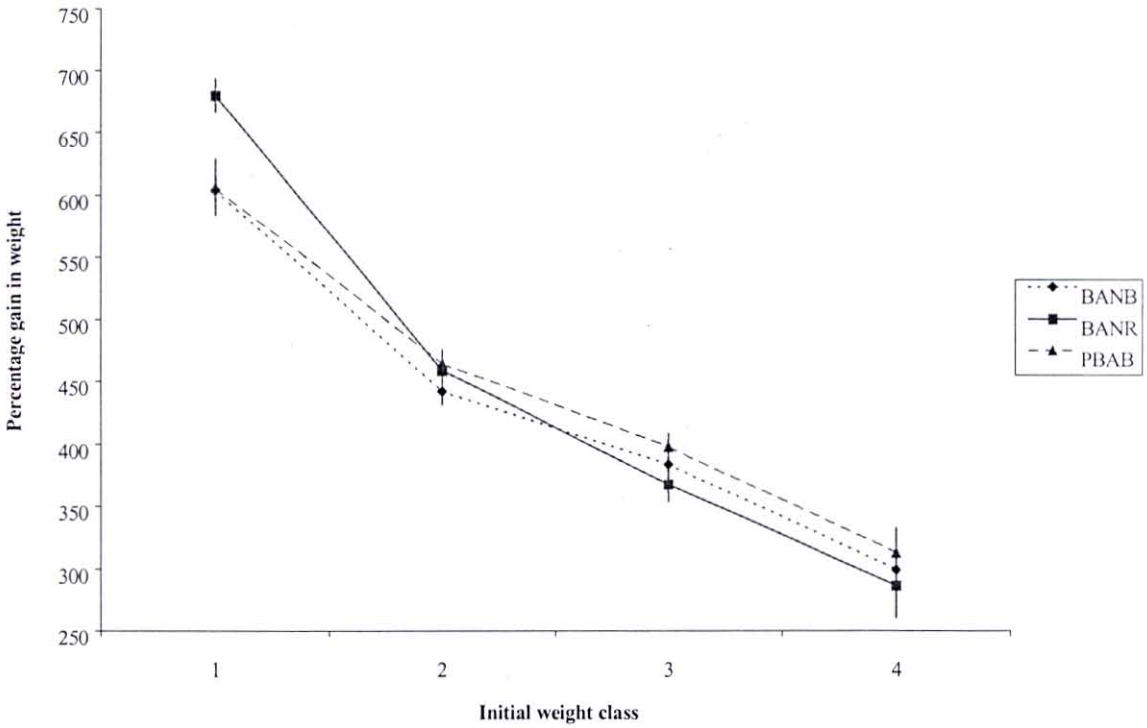
As of January 1999, all the G2 oysters are in outside pools at La Tremblade where they were transferred in October 1998 as decided by the partners during the annual meeting in Galway. 4 full-sib families of the G2.2 will be studied: 2 "good" males and 2 "poor" males crossed with the same female. 100 offspring per family will be reared in individually divided trays (described in the 1st annual progress report) in order to record individual growth curves in a controlled environment up to April 1999, when oysters should weigh at least 5 g. They will then be transferred to L'Houmeau or La Tremblade (according to the progress of the new lab being set up in L'Houmeau) and acclimated to low food availability over 1 month. Non-destructive study of physiology will be made on 30 oysters per family, then these will be dissected and sampled for proteolitic and digestive enzyme activity analyses.

Task 3 : Growth recording

1. Introduction

The results gathered this year in Palavas-Etang de Thau complete the growth analysis that will be done on the G1 generation. The study offers a complimentary viewpoint to that previously obtained with similar oysters in controlled conditions at





Figures 1 and 2. Growth results for Crassostrea gigas raised in the Etang de Thau. The effects of different populations and initial weight classes (1=smallest, 4=largest) Figure 1: Weight gain in grams. Figure 2: specific growth %.). PBAB =Port des Barques, BANB Bonne Anse (Pre-conditioned in Bouin from June 96) and BANR Bonne Anse (Pre-conditioned in Bouin Nov 96). Data represents mean values per rope ± s.e.

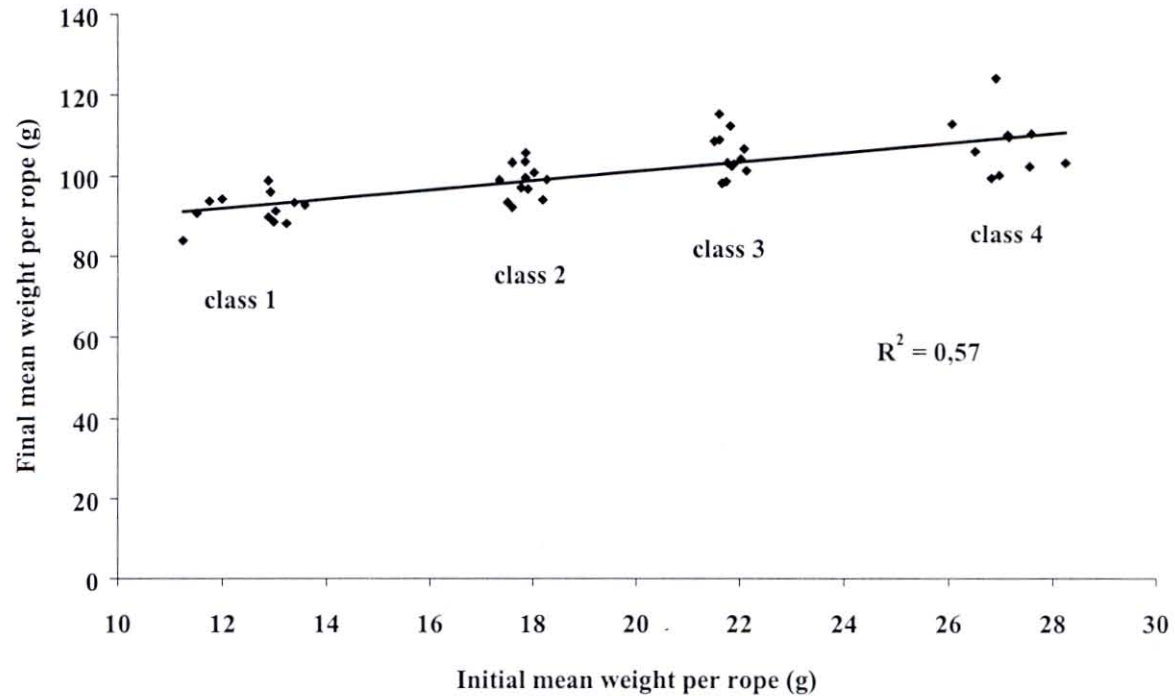


Figure 3. Relationship between initial and final weight in Crassostrea gigas raised in the Etang de Thau. Each point represents a mean value for a rope. The relationship is highly significant and explains 57% of the total variation

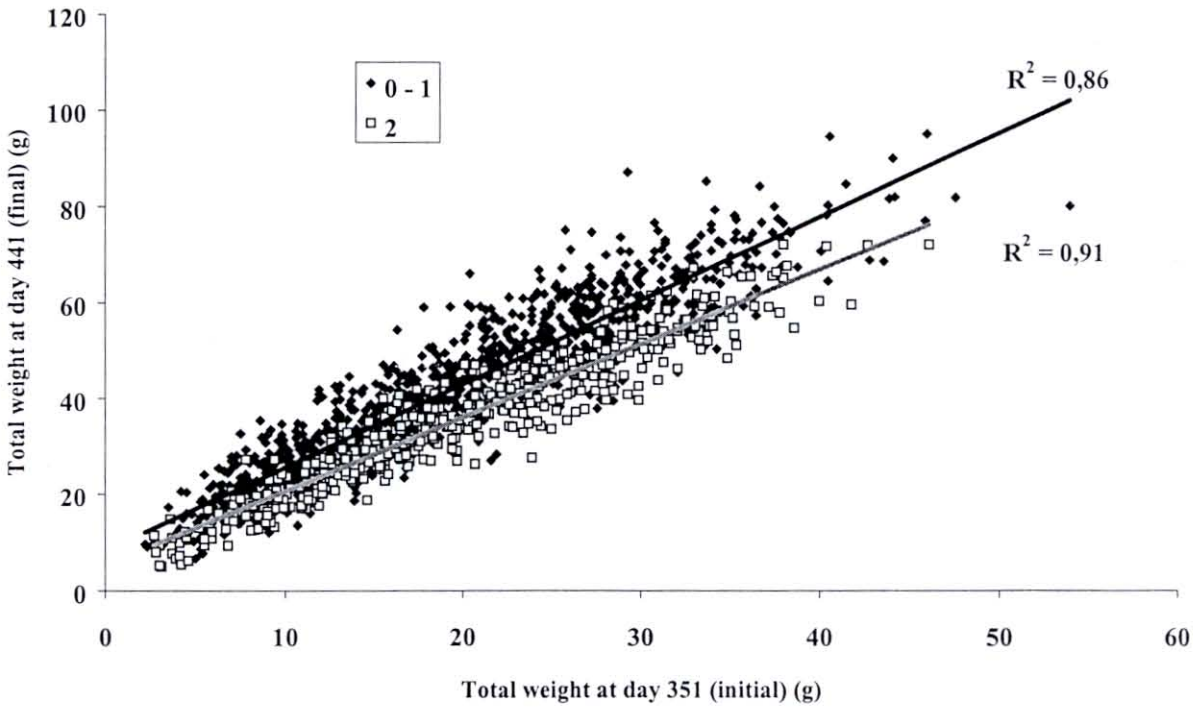


Figure 4. Correlation between total weight at 12 months and total weight at 15 months in Crassostrea gigas raised at Bouin at two density levels (0+1=weak, 2=strong). Points represent individuals (n=1495).

4. Discussion

At this point in the growth experiments the recordings made on the G1 generation are now finished. Comparisons can be made between the populations and also the methods used. The results obtained from the Thau study provide an interesting comparison with those made at Bouin last year (see 2nd annual report). The traditional rope-based farming system probably has an influence on the growth and interaction of the oysters as well as limiting the design of our study to a growth study based on two sets of measurements (initial and final). Some suggestion of genotype x environment effect was shown in that population differences were much more in evidence in Bouin than in Palavas-Etang de Thau.

The results from Bouin last year showed that there is a strong relationship between initial size and growth rate. This relationship was not repeated in the Etang de Thau. Farming methods at Thau are very specific (oysters cemented to ropes) and could affect the ways in which the oysters grow and compete with one another. The rope environment could limit maximum biomass attained. The biggest individuals on a rope would cause greater intraspecific competition than the smaller ones would. Competition from smaller individuals would therefore not influence growth in the size classes 1, 2 and 3, across which the mean growth rate is weakly increasing (figure 1). Gametogenesis could also reduce growth at Thau especially in the largest individuals, whereas at Bouin the experiments were limited to a non reproductive period. Finally, the difference in the initial size - growth rate relationship at Etang de Thau could also be due to ecological effects of other organisms, specific to the area, which attach to an oysters shell and compete for food, a larger oyster would have a larger shell and therefore provide a greater surface and support more competitors, thus confounding the relationship.

Specific growth in Thau oysters had a negative relationship with initial size, thus demonstrating that smaller oysters grow more than the larger ones. The increase in size relative to initial size appeared greater for smaller oysters in the experiment at Thau but not in that at Bouin. The negative relationship with specific growth could also indicate that the larger oysters achieve less growth because they are more inhibited by intraspecific competition in the environment of the rope. This difference in specific growth needs to be examined by making a study of individual growth in the natural environment.

With the final measurements now analysed on these oysters we can see that there is a strong linear relationship between initial weight (1 year old oysters) and final weight (2 year old oysters) at Etang de Thau (figure 3), though this study did not follow individuals (which would be the next logical step). The relationship is not as strong as that observed at Bouin. Growth over the three months in Bouin shows stability for oysters of over a year old indicated by the strong relationship between initial weight (12 months) and final weight (15 months) (figure 4) This stability was unaltered by higher or lower levels of competition. Toro and Newkirk (1990b) found correlations of 82% in *Ostrea chilensis* between weights at the ages of 14 and 22 months. However such correlations are not always so high in the literature. Newkirk (1981) found much weaker relationships in *Ostrea edulis* and concluded that juvenile size was not a good predictor of adult growth. From our previous work with the sieving groups (annual report 1) and on the effects of pre-conditioning of juveniles for the Bouin experiments (annual report 2), we saw that size variation develops very

early during the first months of growth. Then, if uniform conditions are maintained for all individuals, growth rates will be maintained throughout the adult stage.

5. References

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Task 4 Physiological Analyses

Sub-task 4.1 Temporal stability of Physiological characters

1. Introduction

The aim of this subtask was to observe stability through time of physiological performance for individuals of the same age class. Because it was found that the estimates of growth rate using scope for growth (SFG) did not accurately predict individual growth (1st annual report), equipment was conceived to continuously record food and oxygen consumption on individuals. This involved 24 hour measurement periods and is described in the 2nd annual report. These experiments were repeated on the same animals on a total of 5 dates through the 2nd and 3rd reporting periods to give an idea of seasonal variability. The results from the first two measurement dates, September 1997 and October 1997 are described in the 2nd annual report. The present report gives the additional data gathered and a view of the results of the experiment as a whole with its implications for the application of physiological measurements to growth study.

2. Materials and Methods

2.1 Material

The first oysters retained for the temporal stability analysis came from intrapopulation crosses (Arcachon, Bonne Anse and Port des Barques populations) made in April 1996 as described in the 2nd year report. The oysters chosen for the repeated measurements were the Bonne Anse subgroup of these animals. These were used because after mortalities in the spring-summer of 1997, only this group contained enough individuals to be representative.

2.2 Methods

The experimental methods for continuous filtration monitoring and water and biodeposit sampling described in the 2nd year report for experiments in September and October 1997, were repeated in December 1997, February 1998 and April 1998 to augment the seasonal distribution of recordings already made and clarify the picture of the stability of traits through time. The temperatures at the different dates varied, they were: September 20 °C, October 18 °C, December 14 °C, February 10 °C and April 14 °C. Total particulate matter also varied: September 4.39 $mg.l^{-1}$, October 1.95 $mg.l^{-1}$, December 3.85 $mg.l^{-1}$, February 3.51 $mg.l^{-1}$ and April 5.80 $mg.l^{-1}$.

Measurements of physiological traits in these experiments were used to select parents for the G2 generation (see task 2.1) by calculation of the ratio of absorption to respiration (OAR/OCR) over the 5 dates, as an index of physiological performance.

Total weight of each animal was measured at each experimental date and dry weight was estimated using a parallel population, reared in the same conditions, from which individuals of the same total weight were sacrificed and the dry tissue weighed to calculate the allometric relationship with total weight and dry tissue weight.

3. Results

3.1 Growth

The total mean weight in September was 67.1 ± 4.3 g and in April 86.5 ± 5.9 g. Growth followed a linear pattern for all individuals between September and April (figure 1, $p<0.001$, mean $R^2= 0.99$) with a mean growth rate of 86.7 ± 8.0 $mg.j^{-1}$. Size rank of the animals also remained the same during the September-April period ($p<0.001$). A significant increase in dry weight occurred between December, February and April but not between September and December (table 1).

The level of variation in individual weight remained of the same order over the 5 measurement periods changing only from 34.7 % in September to 35.4 % in April. Variation in growth rate was much greater (55.3 %).

	DW		C		PF		I		F		A		OCR	SFG		FTA		RTA	
P	0,0001		0,0001		0,0001		0,0001		0,0115		0,0001		0,2337	0,0001		0,0076		0,0001	
	avr	A	sep	A	avr	A	sep	A	sep	A	sep	A	-	sep	A	dec	A	fev	A
	fev	B	dec	B	sep	A	dec	B	oct	AB	dec	B	-	dec	B	fev	AB	avr	AB
	dec	C	avr	B	dec	B	fev	B	dec	AB	fev	B	-	avr	B	oct	AB	dec	BC
	oct	C	fev	B	fev	BC	oct	B	avr	AB	oct	B	-	oct	B	sep	B	sep	C
	sep	C	oct	B	oct	C	avr	B	fev	B	avr	B	-	fev	B	avr	B	oct	C

Table 1 Analysis of Variance on the effect of date of measurement on physiological parameters and dry weight. All parameters are standardised except FTA and RTA and treated after angular transformation. Results for multiple Student-Newman-Keuls tests are also given: groups with different letters indicate significant differences at the 5 % level. DW-Dry Weight, C-Consumption of particulate matter, PF- Pseudo faeces, I- Ingestion, F-Filtration, A- Absorption, OCR- Oxygen Consumption, SFG-Scope for Growth, FTA- Feeding Time Activity, RTA Respiratory Time Activity.

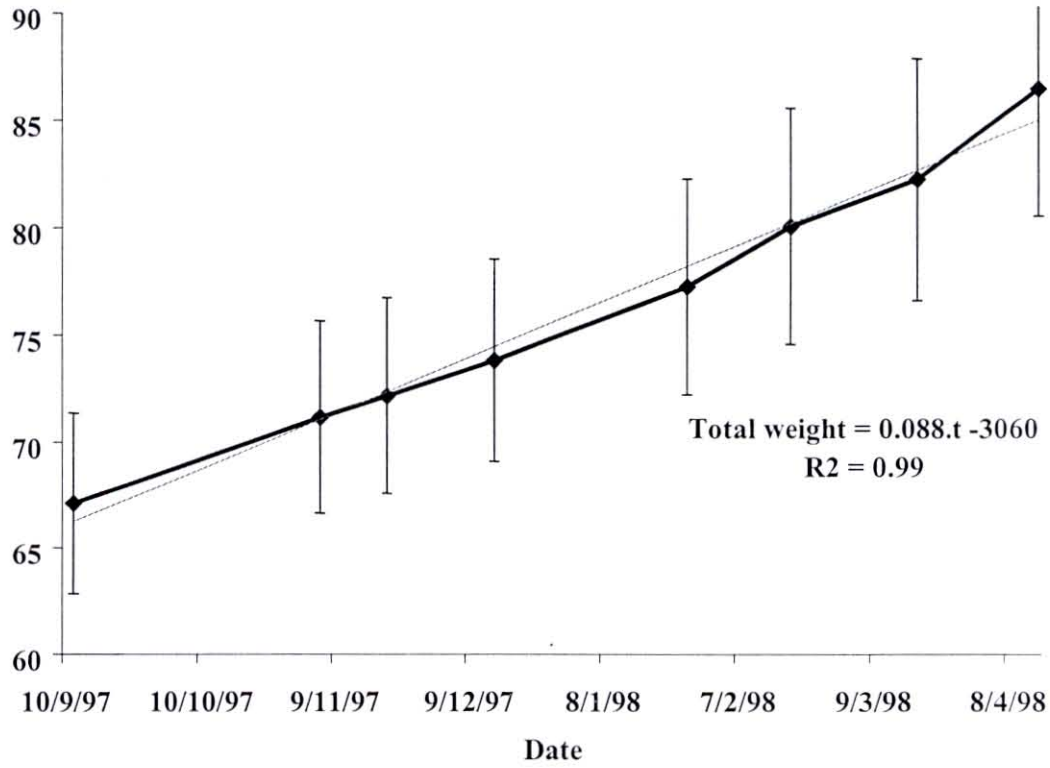

Figure 1 Growth curve based on the means of 30 individuals used for the measurement of physiological parameters over the period September 97 to April 98.

3.2 Allometric relationships

The relationships between total weight and dry weight are very significantly linear ($p<0.001$ for the 5 series). However, the timing of the measurement period significantly affects the relation between the total weight and the dry weight ($p<0.0001$, figure 2).

3.3 Effect of measuring date

Measurement date has a significant effect on both RTA ($p<0.0001$) and FTA ($p<0.008$) (table 1) as well as the components of feeding activity . The changes in mean RTA and FTA across the 5 measurement dates are shown in figure 3. Mean RTA was 64.1 %, 62.2 %, 71.5 %, 77.8 % and 78.7 % respectively in September 97, October 97, December 97, February 98 and April 98. Mean FTA showed a differing pattern: 48.7 %, 65.4 %, 73.5 %, 65.1 % and 70.3 % respectively across the same dates.

No significant correlation was found between RTA and FTA at any of the dates. Variation between individuals was much less for RTA (34 %) than for FTA (46.5 %).

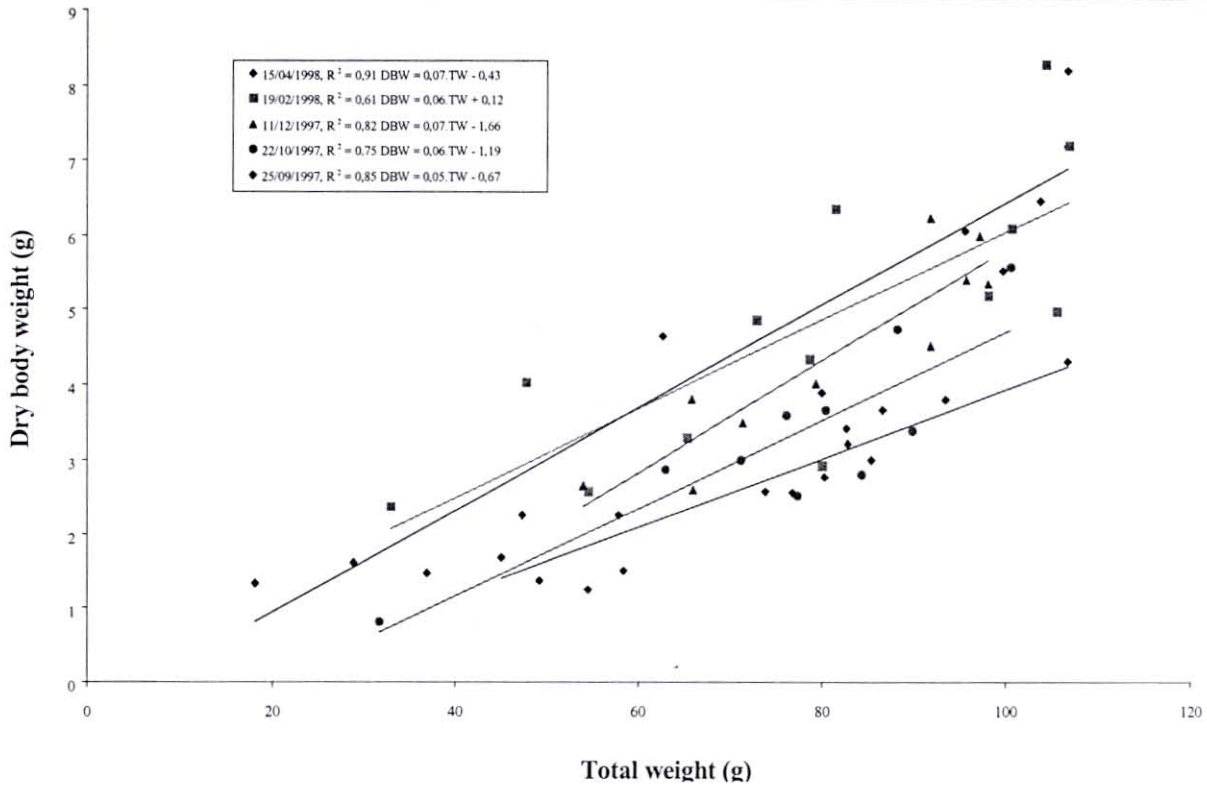

Figure 2 The relationship between total weight and dry weight for individuals at 5 measurement dates from September 1997 to April 1998.

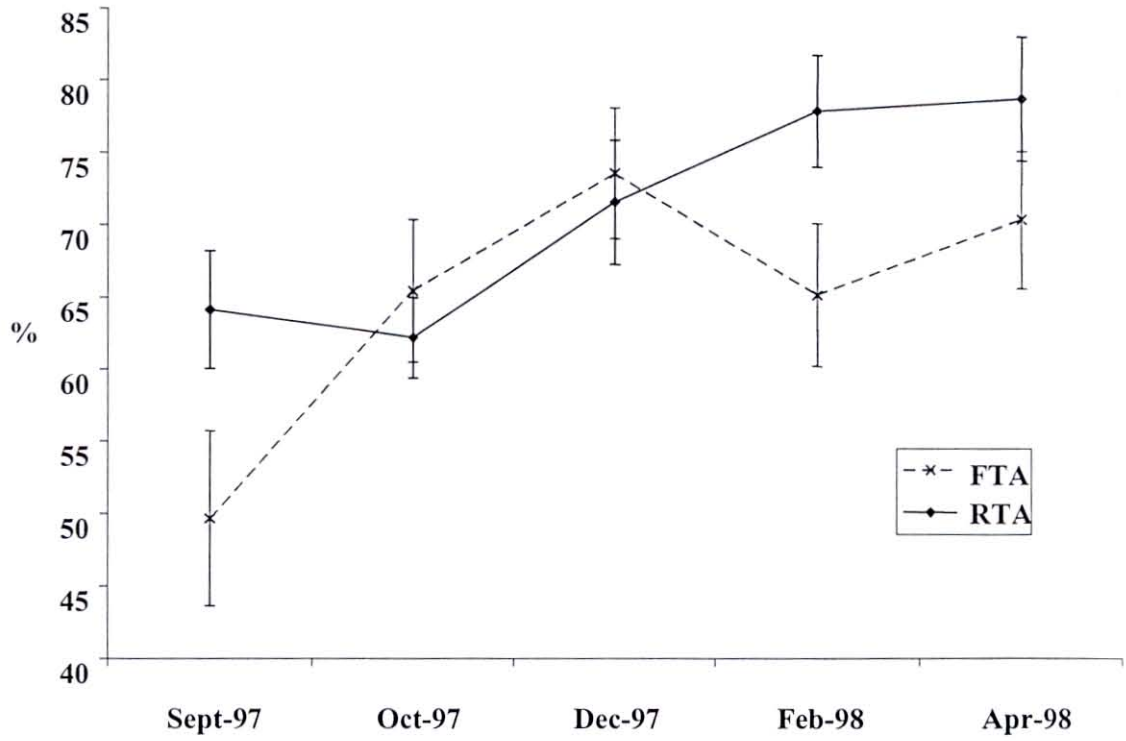

Figure 3 Changes in the level of Respiratory Time Activity (RTA) and Feeding time Activity (RTA) over the 5 measurement dates. The data points represent mean ± s.e.

3.4 Energy use

The quantity of food consumed over the 24 hours varied a great deal between the 5 measurement dates: 90.2 mg in September, 32.6 mg in October, 70.6 mg in December, 44.4 mg in February and 41.1 mg in April.

Physiological parameters were standardised to 1 g dry weight. Their development over time is presented in figures 4 and 5 (analysis in table 1). In October, the quantity of TPM was below the level of pseudofaeces production threshold which itself was so low that practically none was collected. The amount of faeces collected produced over 24 hours varied between the measurement dates, from 5.9 mg in April to 11.3 mg in September. The percentage of absorbed food out of food uptake value is quite similar across the measurement dates from 66 % in October to 76 % in September. In the same way, absorption efficiency went from 0.61 in October to 0.80 in December.

Absorption, oxygen consumption and scope for growth are shown converted to energy terms (J) in figure 4. Scope for growth was positive for all 5 measurement dates and varied from 167 J in October to 1050 J in September.

3.5 Stability over time

A non parametric Friedman test shows that the rank of the animals is conserved over the 5 measurement dates for the principle physiological characteristics (table 2). The ranking of the 30 individuals according to their expected growth at the 5 dates is shown in figure 6. This provides a picture of stability of the individual animals. Two basic types of behaviour were shown, stable and unstable. The stable individuals were sufficiently numerous to give significance in the Friedman test.

Variable	OFR	OAR	OCR	OFRs	OARs	OCRs	FTA	RTA	DW	SFG	SFGs
P	0,0003	0,0006	0,0138	0,0144	0,0113	0,0103	0,0211	0,0210	5.10^{-16}	0,0014	0,0014

Table 2 Results of a Friedman test show that all the following parameters are stable through time: OFR-consummation, OAR-absorption, OCR- Oxygen Consummation Rate, FTA- Filtration: Time Active, RTA Respiration: Time Active, DW-Dry weight SFG-Scope for Growth,. Small s indicates that the parameter was standardised

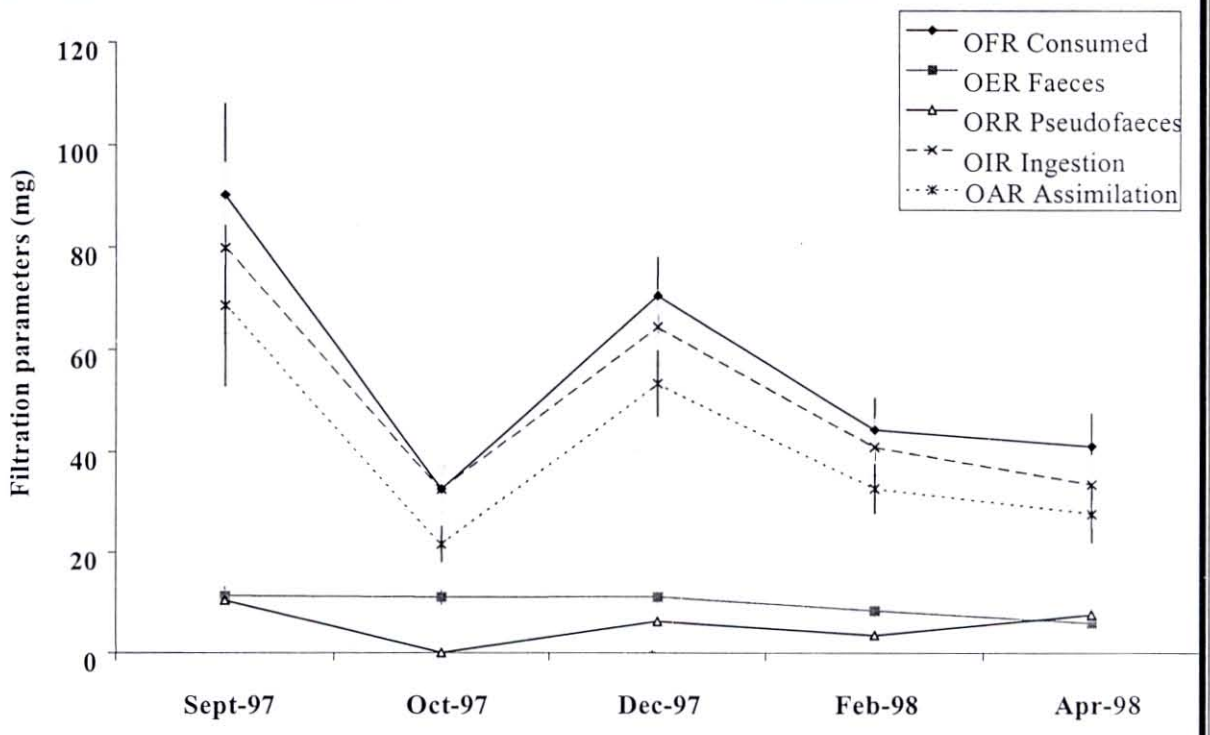

Figure 4 Changes in the quantity of food consumed, ingested and assimilated over 24 hours shown with the quantity of faeces and pseudofaeces produced (mg POM) in the same period for the measurement dates. Data are mean ± s.e.

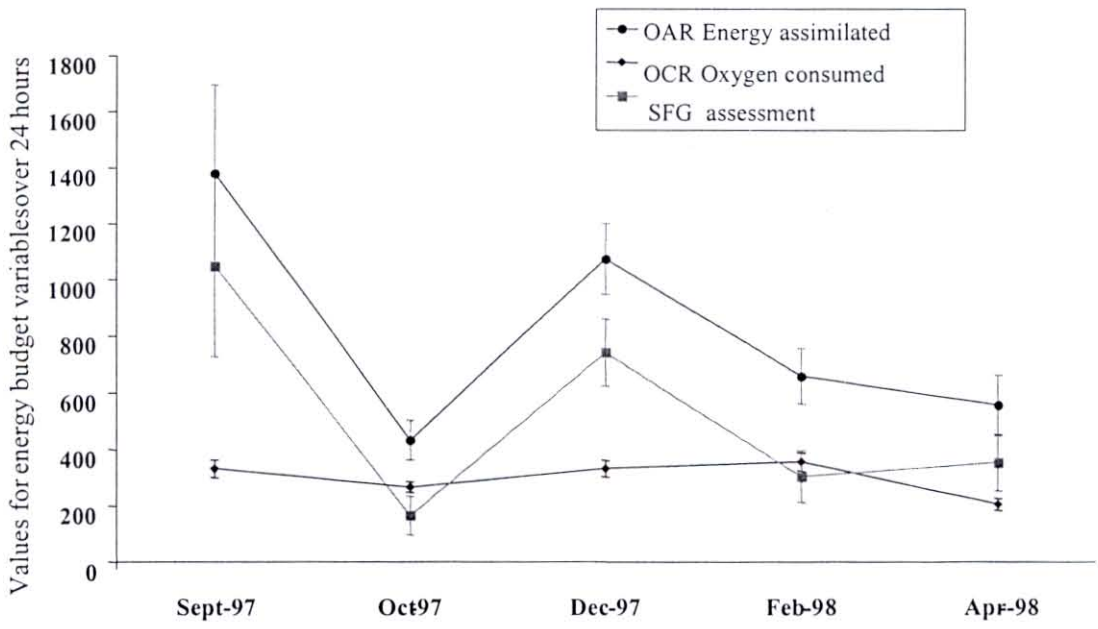

Figure 5 Energy budget for 24 hours at the 5 measurement dates. Data are mean ± s.e.

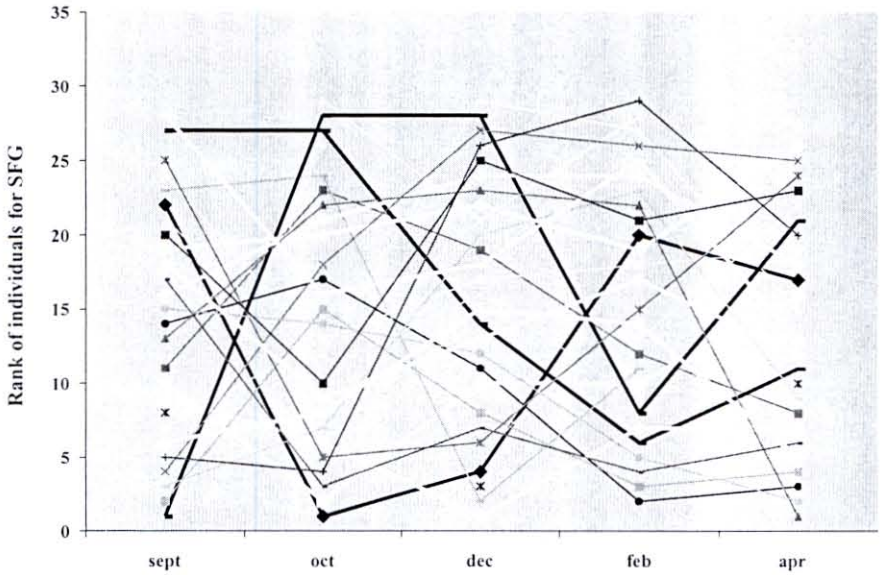

Figure 6 Individual stability, based on predicted growth. Each curve represents an individual. Determination of rank was made according to the SAS procedure PRO RANK, taking into account the ties. The individuals shown with broad dark and pale lines show two different types of behaviour: unstable and stable.

3.6 Classing the individuals

Different approaches were tried to create a synthesis of performance for the 30 individuals taking into account the measurements from the 5 dates. Mean rate of absorption (OARm) and mean rate of oxygen consumption (OCRm) were calculated using the 5 measurements for each individual. The relationship between these two synthesised parameters (OARm/OCRm) was shown to be an effective index for discrimination, varying from 0.21 for the individual with the lowest performance (09B6) to 13.6 for that with the highest (13C7)(figure 7). It should be noted that the individual with the lowest performance had an FTA of zero in October, February and April.

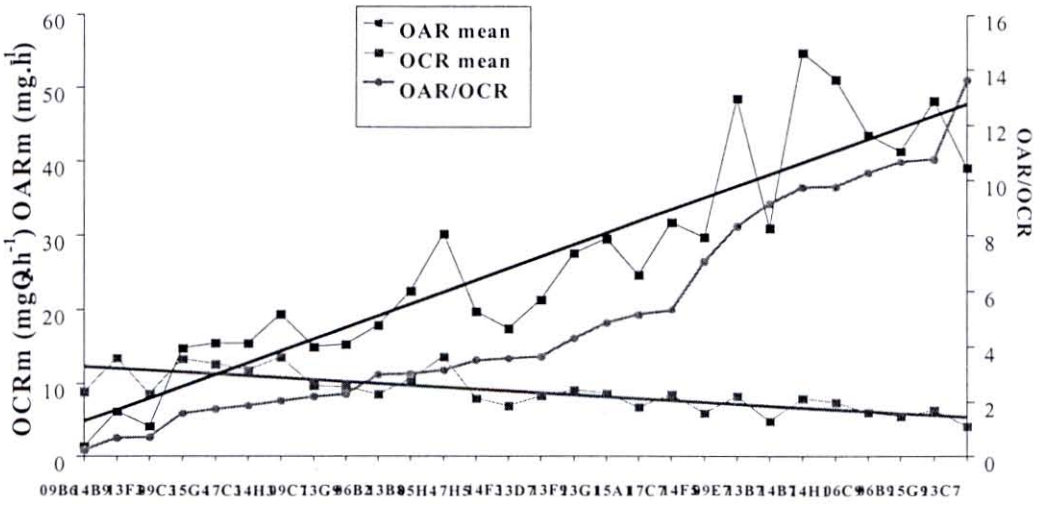

Figure 7 Absorption (OAR) and Oxygen consumption (OCR) for all individuals in the experiment, expressed in quantitative terms and classed according to OAR/OCR ratio.

4. Discussion

The 5 series of measurements were realised under the same conditions. Only the temperature and the quantity of food changed between the measurement dates. The analysis of the effect of the measurement date shows that this had a significant effect on all the physiological characters except oxygen consumption. This result agrees with those in the literature based on observations of natural populations and demonstrates that temperature is the most important factor in explaining seasonal differences (Bougrier et al., 1995; Kuang et al ., 1997; Smaal et al., 1997).

Physiological studies made up until 1996 were based on short separate measurements. Filtration was measured for periods of only 1 to 3 hours, 6 hours maximum. These measurements were then extrapolated to longer periods, usually 24 hours, without taking into account duration of the activity over the whole of the longer period. Our measurement of filtration activity (FA) relied on the simple observation of the animal's behaviour and is therefore not strictly a quantification. Respiratory activity however has been precisely quantified since the definition of RTA by Bougrier *et al.* (1998).

The present work has developed a new system for continuous measurement of filtration in *Crassostrea gigas*. This has allowed the integration of filtration and respiration measurements into energy budget calculations for a marine mollusc for the first time. Definition of a new parameter, FTA (Bougrier et al in prep.), to quantify filtration, represents an advance in the bioenergetic study of molluscs.

The analysis of physiological traits of individuals showed overall stability over the annual cycle under laboratory conditions. However, the statistical test was made on rank of value rather than on raw data. It is difficult to present such variable trends graphically (figure 6). The presence of such instability could be due to non repeatability in the measurement method, unpredictable behaviour in the individuals studied, or its dependant on factors that we were unable to control, *e.g.* atmospheric

pressure. Physiological parameters are extremely dependant on environmental conditions, in our experiment, temperature and food level fluctuated between measurement dates and food level also varied within measurement periods. Self-regulating equipment to regulate food supply level, such as in Winter (1973) or Riisgård and Møhlenberg (1979), would enable a more rigorous control of this factor for the comparison of individuals.

It is possible that sub-groups, with different tolerance to environmental variability, exist within the 30 individuals despite the fact that they all originally came from the same population. In figure 6 the stable and unstable individuals can be distinguished although the Friedman test indicated overall stability (due to the large number of stable individuals). The difference in relation of individuals to their environment could be due to differing sensitivity to stress among individuals of the same age and from the same cross. Certain species have the same behaviour in relation to temperature. Stenothermic species only tolerate small changes in temperature whilst eurythermic species have physiological functions that vary very little despite large changes in temperature in their environment. Genetic analysis may be able to offer some answers by analogy with species where genetics explains differences in behaviour (Whiteley *et al.,* 1997; Loughna and Goldspink, 1985).

The major problem in the estimation of filtration and oxygen consumption is the lack of knowledge about periodicity. Over 24 hours, no periodic pattern was shown for RTA (Bougrier *et al.*1998). However 24 hours may be insufficient for efficient and repeatable estimation of an animal's overall activity. Activity is very probably affected by factors that cant be practically controlled and, though this hasn't been demonstrated experimentally, oyster behaviour probably depends on more than just temperature and salinity of the water. Similar experiments made on *Ostrea edulis*, the European flat oyster, showed that external stress factors, such as vibration and variation in light intensity can greatly perturb overall activity, by causing the valves to close, but this species is well known to be more sensitive to stress than the japanese oyster. Atmospheric pressure can also play an important role in stress on the animals and their tendency to close their valves. Activity is in general very sensitive to climatic conditions, particularly in stormy conditions when there is considerable variation in pressure.

It is not yet possible to measure oxygen consumption and filtration activity simultaneously because the measurement systems require different flow rates. Study of correlation between these traits is therefore difficult. Additionally, given the high sensitivity of bivalves to external factors, estimation of growth potential could vary from day to day. Open measuring systems that enable both oxygen consumption and filtration monitoring have already been used successfully with fish (Blazka *et al* 1960) and could be adapted for studying marine bivalves. A closed system recently described by Erikson and Iverson (1997) and allowing simultaneous measurement of filtration and oxygen consumption, could also represent a solution.

For the selection of genitors for the G2 generation, an overall index of physiological performance was needed. This had to be selected carefully because of the differences between the values recorded on the 5 measurement dates did not present a very clean-cut picture. Our index (figure 7) integrated the different activities and was based on the means of the 5 measurement dates. This classification favoured the stable individuals. Unstable individual tend to have an intermediate index whilst stable individuals have either a high or a low index depending on their performance. The choice of genitors therefore took this

characteristic into account, and individuals with extreme index values were chosen.

Individuals with high performance have both high absorption and low oxygen consumption, indicating the value of using the performance index for the selection of genitors. High performance individuals have the double characteristic of high energy acquisition and low metabolic costs.

In conclusion, the index summarises the 5 measurements, taking into account filtration and oxygen consumption and the level of their stability. The aim is to produce full-sib families for G2 from genitors with the most contrasting physiology.

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Sub-task 4.2 Non destructive measurements of the physiological functions

1. Introduction

Physiological parameters: oxygen consumption, filtration rate, and absorption were measured on a group of 68 adult oysters from three populations (Arcachon, Bonne Anse and Port des Barques). These same animals were later given to other partners to record other physiological (tasks 4.3 and 4.4) and genetical aspects (tasks 5.1 and 5.2). In the final collection and synthesis of data, the relationships between the measured statistics will be examined.

2. Materials and methods

Experiments were conducted in the second reporting period and experiments are described in detail in the second progress report. Here we include a fuller examination of the results found in this part of the study.

3. Results and Discussion

The present experiment enabled the physiological comparison of animals of different size and population on an experimental sample with a common age. The populations were found to be significantly different in size (2nd progress report) but did not show significant differences in physiological parameters measured (table 1).

Most physiological functions are dependant on an individuals size. Allometric models are considered best for describing the variation of oxygen consumption or filtration with weight. However, values for these activities vary considerably with stage of development (Gerdes 1983 a, b) as well as between observations made by different authors on the same stage (Bayne and Newell 1983). These studies were nearly all based on natural populations and therefore on groups of mixed age, so it is difficult to know if the variation observed was due to age, or size, or both. Importance of stage of development was demonstrated by the finding that allometric coefficients for oxygen consumption were similar for free-swimming larvae and juveniles in the mussel *Mytilus edulis* but different for adults (Hamburger et al. 1983). This group also found that within any given stage, allometric relationships followed a linear pattern.

The results of the present study clearly show a linear relationship between oxygen consumption and dry weight, under our experimental conditions (2nd year progress report). This result is important because it is the first time that the consumption of a small individual has been shown to be proportionally the same as that of a large one. Exponential models of individuals from the natural environment

had previously predicted that small animals would consume proportionally more than larger ones. Recent adaptations to classic exponential models do not improve the fit to our results (Bougrier *et al.* 1995, figure 1). Several studies made on hatchery produced spat, have already shown atypical allometric relationships for consumption and ingestion (Strickland and Dabinett, 1993).

The linear pattern observed could be due to the very low seston load of the water. 15 days acclimation would have been enough to change the relationship normally observed under high food availability.

	ARC	BA	PDB	P
TW (g)	25,900 ± 2,899[a]	36,784 ± 3,279[b]	35,784 ± 2,646[b]	0,020
CR ($l.h^{-1}$)	1,332 ± 0,153	1,518 ± 0,227	1,232 ± 0,154	0,532
FR ($mg.h^{-1}$)	2,103 ± 0,243	2,493 ± 0,371	2,162 ± 0,333	0,647
ER ($mg.h^{-1}$)	1,702 ± 0,203	1,927 ± 0,302	1,797 ± 0,269	0,827
IR ($mg.h^{-1}$)	2,103 ± 0,243	2,493 ± 0,371	2,162 ± 0,333	0,647
AR ($mg.h^{-1}$)	0,401 ± 0,078	0,566 ± 0,119	0,364 ± 0,073	0,270
OCR ($mg\ O_2.h^{-1}$)	1,282 ± 0,105	1,31 ± 0,094	1,032 ± 0,047	0,053
SFG1 ($J.h^{-1}$)	-10,067 ± 2,404	-7,128 ± 2,924	-7,262 ± 1,610	0,619
SFG2 ($J.h^{-1}$)	-7,649 ± 2,242	-5,123 ± 2,853	-5,674 ± 1,600	0,716

Table 1 Population effect on standardised physiological parameters. SFG1 is calculated assuming that the animal is 100% active for both filtration and oxygen consumption. SFG2 is calculated with RTA (Respiratory time activity)=86% and active filtration estimated by the length of time the values were observed open. Exponent letters indicate the significantly different groups (5% level). Data are mean ± s.e. ARC=Arcachon, BA=Bonne Anse, PDB=Port des Barques, TW = Total weight, CR= Clearance rate, FR= Filtration rate, ER= Egestion rate, IR= Ingestion rate, AR= Absorption rate, OCR= Oxygen consumption rate, SFG= Scope for growth.

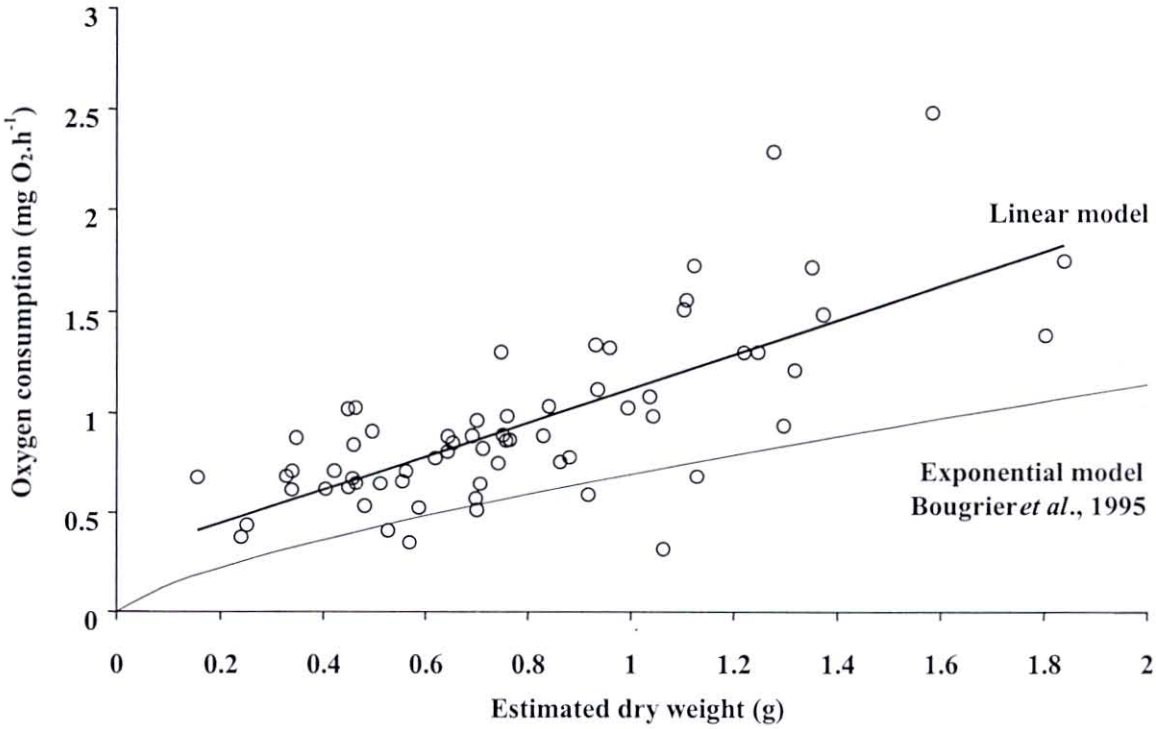

Figure 1. The relationship of oxygen consumption rate and estimated dry weight . The regression is significantly linear ($p<0.0001$) and explains 53% of the variability. The exponential model conceived by Bougrier et al. is presented alongside (calculated for temperature = 15°C).

The relationship between absorption and size shows considerable variation between individuals (figure 2). One hypothesis that could explain this wide distribution is the existence of two subgroups with different feeding behaviour. It is impossible to test this hypothesis statistically with the present data, but the trend is clear. The two subgroups could be distinguished by their specific allometric patterns, one could be typically exponential, the other linear, as observed for oxygen consumption (figure 2). The population which had initially been maintained in optimal food conditions, had been acclimated only 15 days in conditions with a limited food supply. The sub-group effect observed could be due to insufficient acclimation of a part of the population during these 15 days. One sub-group which had already adjusted would show the linear relationship, while that which had not would show an exponential one. If this were so, it indicates that acclimation takes place faster for oxygen consumption, where an overall linear relationship was found, than for absorption, where only some of the individuals appear to follow this pattern.

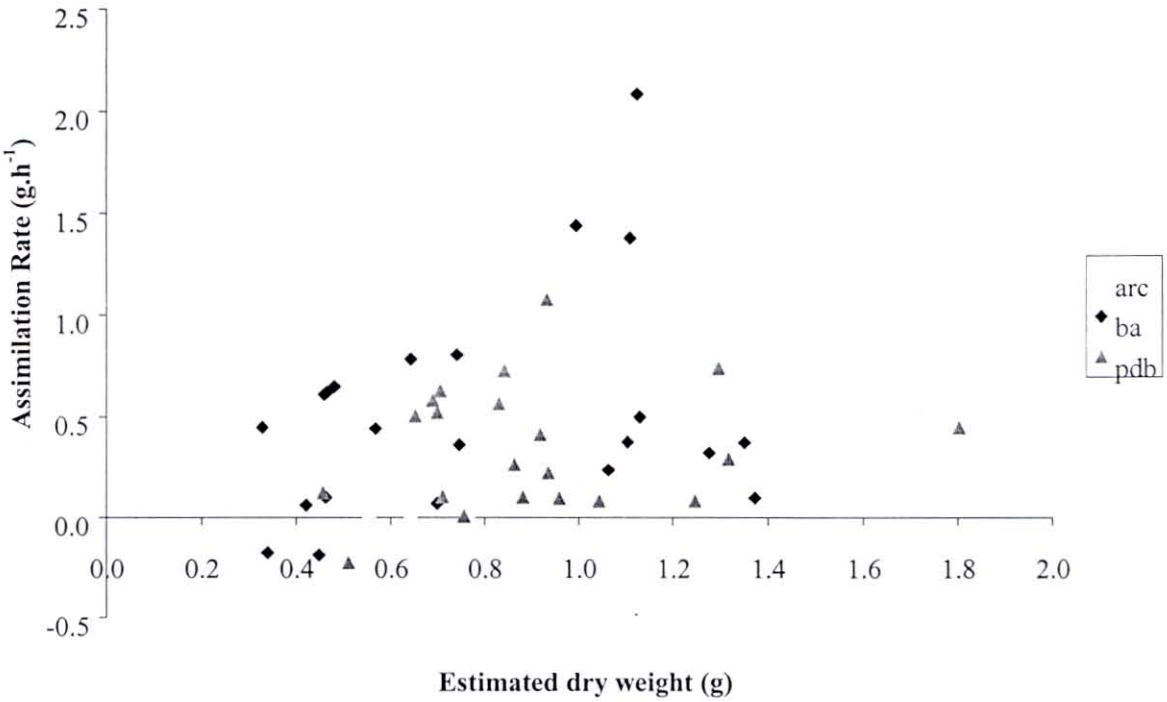

Figure 2. Variation in 'absorption rate' with size in Crassostrea gigas. arc= Arcachon, ba= Bonne Anse, pdb= Port des Barques. Existence of two sub-groups could explain the variability between individuals.

Scope for growth is negative for many individuals in this experiment (figure 3). This shows that the experimental conditions (2 mg.l^{-1}TPM at 15°C) caused a stress situation and the animals had started to use their reserves. However the period of study was too short to detect any decrease in body weight or mortalities.

The relation between scope for growth and dry weight is significantly linear and negative (p=0.009, figure 3). This relationship shows that small individuals are less affected by limited food conditions than large individuals. The high degree of variability between individuals could be due to the existence of sub groups with different relationships between scope for growth and dry weight, one group positive, one group negative. Scope for growth represents theoretical growth per gram dry weight which is the same as theoretical specific growth. In task 3, and in an examination of juvenile growth (day 91-day 147) with animals from the same populations as the present experiment (Collet, 1998), specific growth was shown to have a negative relationship with initial weight. Small individuals had a relatively larger gain in weight than large individuals. Scope for growth gave the same negative relationship with estimated dry weight.

An index of 'physiological performance' can be quantified by calculating the ratio of energy obtained to that expended, standardising results to an animal of 1g weight. Figure 4 presents the values of the ratio AR/OCR (Absorption Rate/Oxygen Consumption Rate) for all the animals in the experiment in rank order with the corresponding standardised values of AR and OCR expressed in energy terms. Absorption increases in a quasi-linear fashion for individuals with a low or medium AR/OCR ratio. However, for animals with a high ratio, the linear pattern disappears. Oxygen consumption does not appear to follow a trend with the AR/OCR ratio.

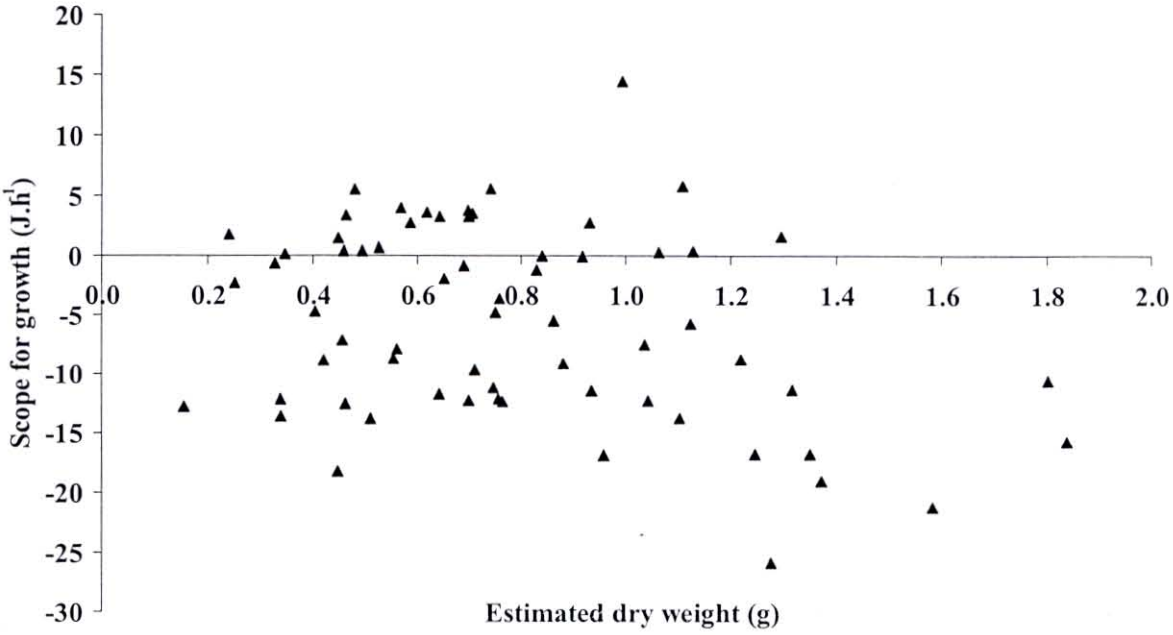

Figure 3. The relationship of scope for growth with estimated dry weight . The regression is significantly linear and negative (p =0.009)

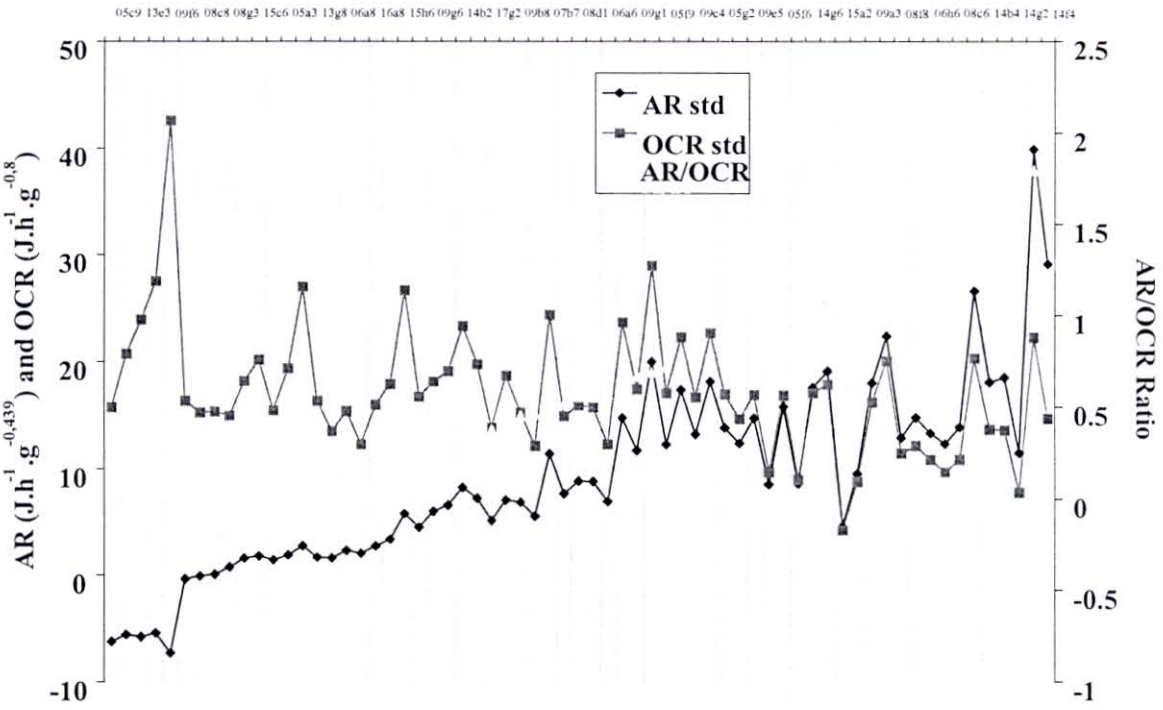

Figure 4. Absorption rate (AR) and Oxygen consumption rate (OCR) for all individuals, standardised for individuals of 1g, expressed in terms of energy and classed according to rank of AR/OCR ratio.

In this experiment, if absorption tends to increase as respiration decreases, such a result is confirmed in the experiment in task 4.1; the individuals with the best growth performance absorb more overall but expend less energy. This indicates the same trend as previous studies made on the relationship of physiology and heterozygosity (Garton *et al.* 1984, Hawkins, 1995, Toro *et al.* 1996a) where higher performance of heterozygotes is explained by higher ingestion and lower energy expenditure.

Working in a non-destructive manner on an ecophysiological project presents a problem for data standardisation. The relationship between dry tissue weight and total weight was therefore established using a population maintained under identical conditions to those studied for the physiological parameters. This relationship is very significantly linear (2nd progress report) as is often the case in laboratory raised animals. However, the cross significantly affects the parameters of this relationship. This can be explained by differences in growth rates observed between the populations. It is already known that growth rate modifies the condition index in *Crassostrea virginica* and *Argopecten iradians iradians* (Rheault and Rice 1996).

Visual estimation of filtration activity is not sufficiently accurate for the correction of estimates of physiological parameters, the automated method used in task 4.1 is much better (Bougrier *et al.* in prep). In addition, the activity of oxygen consumption was not measured directly on individuals. The RTA value used in the calculations (86%, Bougrier *et al* 1998) was applied to all individuals and it is possible that a high degree of variation exists between these individuals and influences the value of scope for growth.

The task 4.2 investigation will be continued with animals of the G2 generation in 1999.

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Sub-task 4.4 Measurement of the digestive enzyme activity

1. Introduction

In 1997, we modeled absorption efficiency using a Michaelis Menten-like expression combining relative ingestion rate, and oyster specific digestive enzyme activities (amylase and laminarinase). More than 50% of the variance was explained by this model and two different physiological constants were obtained characterizing two subgroups in the 60 samples analysed from the June experiment these were named KM1 and KM2.
For each subgroup, these physiological constants characterize the value of the relative ingestion rate where absorption efficiency (per digestive enzyme unit) is half its maximum value. One subgroup had a high absorption efficiency at low ingestion rate, while the other had the same maximum absorption efficiency value only when ingestion rate was high.
In the conclusion of the last report we planned to determine the significance of these physiological constants. Among the possible hypotheses, we suggested to look
- 1/ at the Michaelis constant values of these enzymatic activities in the two sets of samples
- 2/ at the genetic level, as amylase is coded by at least two different genes in *C.gigas* oysters differently represented in individuals
- 3 / enlarge this set of data by looking at the same population in a different ecosystem.

1998 activities were focused on these 3 objectives :

- Km of amylase and laminarinase of available samples among the 60 'June 97' ones were determined.
- PCRs were performed on genomic DNA extracts of these samples (provided by Dr Kotoulas, partner 5 from Greece) using adapted primers.
- A set of G1 oysters placed in Thau was also sampled in spring 1998 to measure performances and digestive activities in this special trophic situation and specific activities are under analysis.
- Sampling of G2 young larval stages, previously planned for the project was delayed, because it seemed a priority to understand previously obtained results on broodstock more profoundly first.

2. Materials and Methods

Km determination : 25 samples containing sufficient digestive gland extract were used for laminarinase and amylase Km determination. 5 concentrations of substrate were tested (0.1 ; 0.2 ; 0.3 ; 0.4 ; 0.5 ; 0.6 ; 0.8 ; 1 mg/mL for amylase, 0.025 ; 0.04 ; 0.1 ; 0.2 ; 0.5 ; 1 ; 2 mg/mL for laminarinase) and a Lineweaver-Burk treatment of the data allowed determination of Km values.

Genetic analyses : 68 samples were analyzed using the PCR technique and amylase primers AMYHO and HAMY2 adapted to obtain two genomic variants of 2000 and 2500 bp length.(See annex 1). The PCR protocole was 94°C, 30s denaturation,

58°C, 60s annealing, 72°C, 90s elongation, 35 cycles. Products were analyzed after electrophoresis on 1% agar gels and BET (direct UV observations and photos, or after transfer onto a nylon membrane and hybridization using an amylase P32 labelled probe). A second PCR was performed using AMYH4/HAMY1 primers (smaller fragment of 200bp) to test quality of the DNA, when no amplification was obtained using the first set of probes.

Thau samples : 20 oysters per size class, four size classs and three origins were collected by the Ifremer team at Thau (D.Buestel *et al.*). As a preliminary step we analyzed 40 individuals from four size classes (10 per class : 0-16, 16-20, 20-24, over 24g at the beginning of the transfer to Thau) and from one origin : Port des Barques raised in Bouin. Each oyster was weighed and measured in length, before dissection of the digestive gland, which was also weighed, (as were the rest of the tissues), then frozen in liquid nitrogen. Then samples were prepared as previously described for digestive enzyme analyses. A part of the mantle was sampled and preserved for genomic DNA extraction and analyses.

3 Results: 3.1 June experiment (Genephys 97):

3.1.1 Characterization of amylase and laminarinase Km values.

24 individuals were selected among the 68 samples according to their clear assignment to each of the two subgroups described before. After determination of hydrolysis rates, against substrate concentrations, Lineweaver-Burk treatment (1/V versus 1/S) demonstrated linear regressions with R^2 over 99% for amylase Km and 70% for laminarinase Km.

Amylase Km varied from 0.34 to 1.09 mg/ml with a mean and SD respectively 0.74, 0.17 mg/ml for 24 individuals. Laminarinase Km varied from 0.020 to 0.120 mg/ml with a mean and Sd 0.060, 0.018 mg/ml.
Among these data, 10 samples originated from the subgroup demonstrating a low physiological constant (KM1) and 14 from the subgroup demonstrating a high KM2physiological constant.
The means for the two corresponding catalytic constants for amylase were not significantly different (respectively Group KM1 : 0,77 SD 0.15 and Group KM2 : 0,71 SD 0.19). The same result was obtained with laminarinase (Pop KM1 : Km 0,049 SD 0.020 and Pop KM2 : Km 0,058 SD 0.016). Scattering of the data was larger for laminarinase Km, and for subgroup KM2 for amylase (Figure.1).

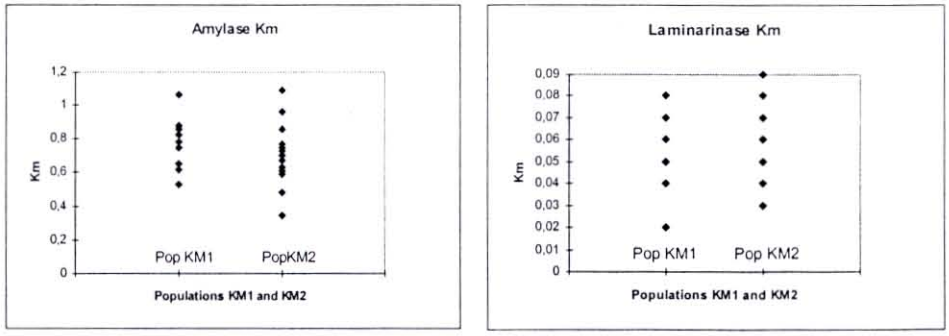


Figure 1: distribution of amylase (left) and laminarinase (right) Km values in the two subgroups KM1 and KM2.

3.1.2 Relationships between amylase and laminarinase Km and absorption efficiency :

On the total samples, absorption efficiency varied significantly (P=0.05) with Km values for amylase activities, but not for laminarinase (Figure.2). For this enzyme, a part of the data had this trend, but the dispersion was too high to have a statistical estimation. When data of subgroups KM1 and KM2 were separated, trends were better observed especially for laminarinase, in the subgroup KM2.

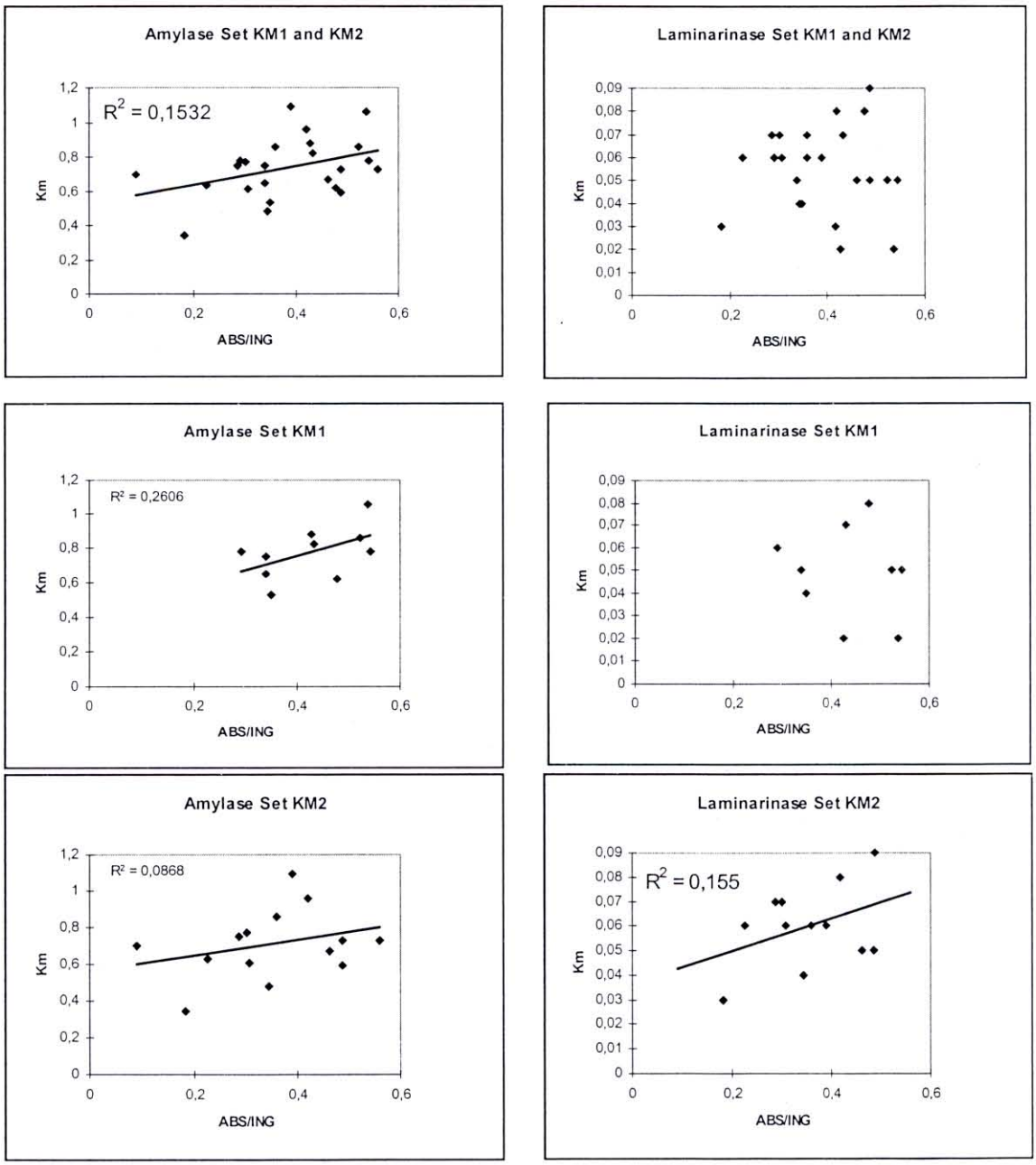


Figure 2: Relationship between absorption efficiency and Km of amylase (left) or laminarinase (right) of all the analysed samples. Upper graphs : total samples ; medium : subgroup KM1 ; lower graphs : subgroup KM2

3.1.3 Relationship between amylase and laminarinase Km and Relative Growth Rate

Again using all the data (Figure 3), significant relationships were shown between Relative Growth Rate (RGR) and amylase Km (p=0.05). Some of the data could have the same trend in laminarinase Km. When KM1 and KM2 data were selected separately, trends were only observed for set KM2. It was highly significant for amylase (p=0.01), but not for laminarinase. No trend was observed in set KM1 where the data were poorly dispersed.

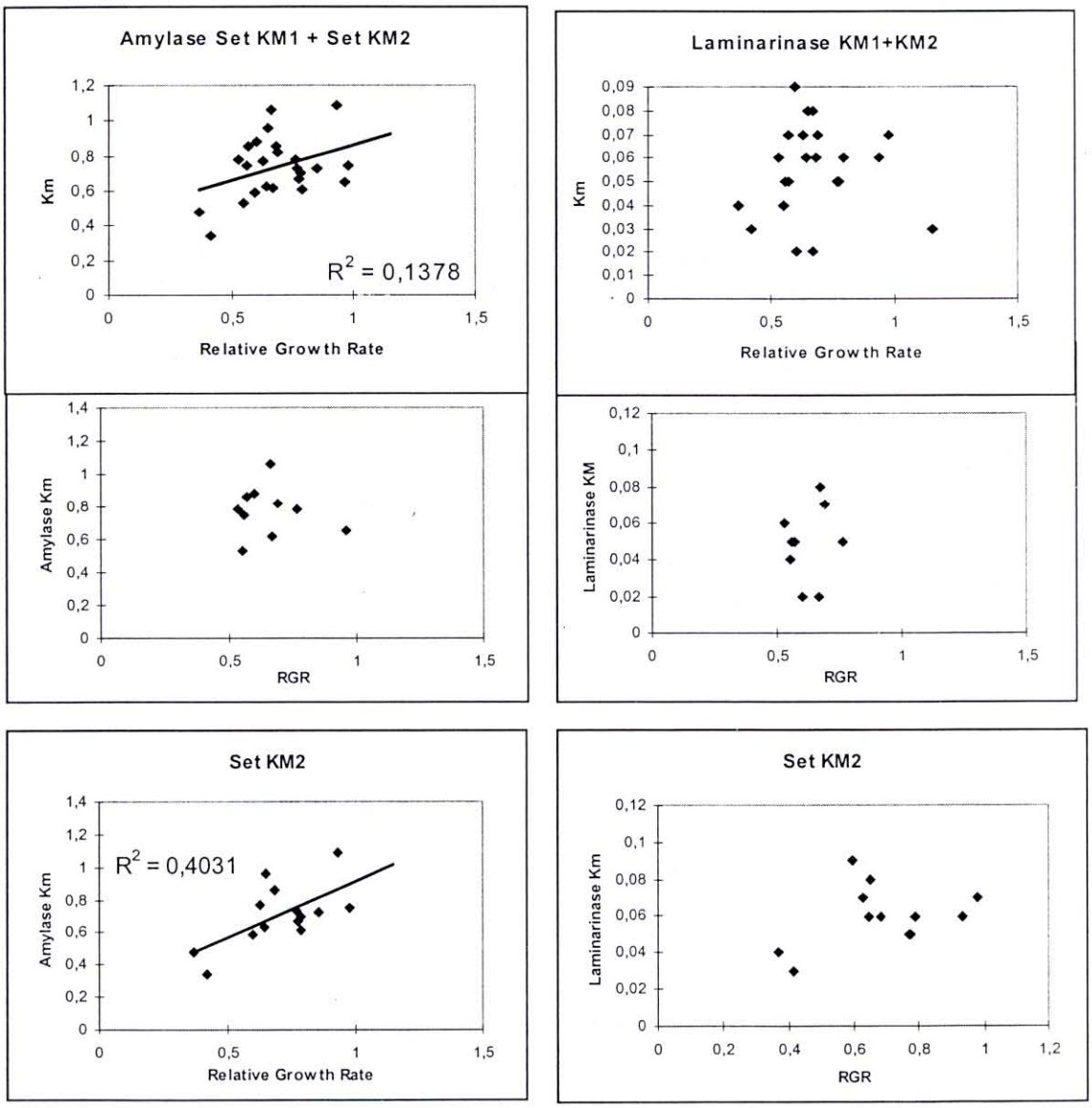


Figure.3: Relationship between Relative Growth rate and Km of amylase (left) or laminarinase (right) of all the analyzed samples (upper graphs) ; medium graphs : Set KM1, lower graphs : Set KM2.

4 Discussion: 4.1 June experiment (Genephys 97)

Km values for the two enzymes varied accross a large range with maximum values observed being 3 to 6 times greater than the lowest ones in a similar range of the two observed physiological constants KM1 and KM2 (0,18 and 0,86). But the means for the two groups were identical. As a consequence the enzymatic Michaelis constant values varied in a same range as physiological constants, but their mean cannot fully explain the differences in the physiological constant values.
The large scattering of the data for laminarinase Km is related to the poor regression lines obtained using the Lineweaver-Burk treatment for laminarinase Km determination (R^2= 0.70) So we will give laminarinase Km values as an indication. Km determination for this enzyme should be improved. In contrast, regression lines for amylase Km determination were excellent (R^2= 0.99), and the higher scattering observed for subgroup KM2 can be considered as a biological result. So in the next results, we will focus more on amylase Km than on laminarinase Km in the statistical interpretations.

Similar significant correlations were observed between amylase Km values, absorption efficiency and relative growth rate on the total set of data, suggesting that Michaelis constant can explain a part of the variance in absorption efficiency and relative growth rate.
An interesting result is shown when the two subgroups are considered separately : a higher significant relationship is observed between amylase Km and relative growth rate in the KM2 subgroup. Similar trends are observed for absorption efficiency, and this selection of data also reveals similar trends for laminarinase
These results suggest that the two physiological constants previously shown, separated two subgroups which also had different relationships between the Michaelis constants of the two digestive enzymes and absorption and growth performances. Subgroup KM1 would be independant for enzymatic Km values.

The large distribution of amylase Km values in the subgroup KM2 could be due to a mixture of different amylase proteins corresponding to different genes and their allelic polymorphism, as subgroup KM1 would have less protein diversity, and so less changes in the sequences of the corresponding amylase genes, or genetic changes sufficiently large that no active protein was expressed.
In parallel work, we had determined the sequence of one cDNA of amylase in *C.gigas*. So we chose two primers corresponding to the major part of this cDNA, and did PCR on genomic DNA of all the corresponding samples, to have an idea of a possible polymorphism at the level of amylase gene(s).

3 Results: 3.2 Genetic analyses

3.2.1 PCR on genomic DNA

UV analyses of PCR products using primers AMYH0/HAMY2 and genomic DNA from the oyster samples (prepared by Dr Kotoulas (Greece)), demonstrated two bands at about 2000 and 2500 bp. Hybridization using an amylase probe was positive for these two bands (see Annex 2 of this section).

However, some samples demonstrated only one band and others no band at all. These last samples were PCR using another set of primers (AMYH4/HAMY1) corresponding to a smaller part of the gene, and positive amplifications were obtained demonstrating that the amylase DNA was present in the extract in sufficient quantity for the amplification procedure. We found that the 2500bp band intensity was sometimes lower than the intensity of the 2000 bp band in some samples. So we classified the samples into four sets numbered 1 to 4, corresponding to the different patterns observed (Table 1). Categories 1 and 2 were present at equal frequency (17% of the population) and categories 3 and 4 both had an equal higher frequency (33%).

Category	1	2	3	4
PCR Response	— —	– – —	—	
Number of samples observed with this pattern	11	19	20	10
%	17%	33%	33%	17%

Table 1 : number and percentage of samples in each genetic category

3.2.2: Genetic pattern, amylase and laminarinase Km values

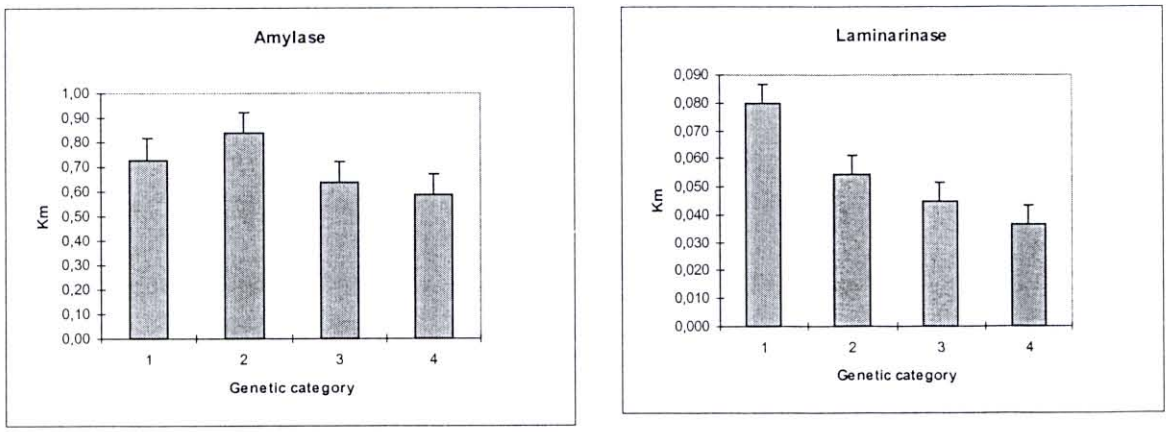


Figure 4: Mean and confidence interval (p=0,05) of amylase Km (left) or laminarinase Km (right) for each genetic pattern classified in four categories.

In Figure 4, a trend was observed with the highest amylase or laminarinase Km values for categories demonstrating two bands (categories 1 and 2) compared to categories with one or no band (categories 3 and 4). When categories 1 and 2 were pooled, their mean Km was significantly higher (T test p=0,01) than pooled categories 3 and 4 for the two enzymes.

3.2.3 : Genetic pattern and Relative Growth Rate

Means of relative growth rates for these categories were significant between group 1 or 2 and group 3 (Student test p=0.01), but not significantly different from group 4, because category 4 had a high standard deviation. But when distribution of the data are considered, it is clearly shown that a few data from the category 4 were outside of the general trend (Figure.5).

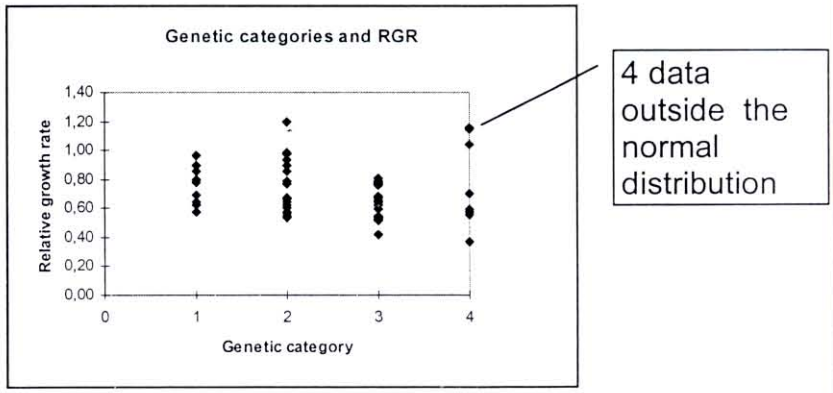


Figure.5: Relative growth rate and genetic categories.

3.2.4: Genetic pattern and the two subgroups KM1 and KM2 :

Distribution of genetic categories between the two subgroups KM1 and KM2 demonstrated a non significant (khi^2=1.63) higher percentage of samples from genetic category 4 in the KM1 subgroup and of samples from genetic category 1 in the KM2 subgroup, categories 2 and 3 being similar (Table 2).

Genetic category	Subgroup KM1	Subgroup KM2	Total samples
1	13%	25%	11
2	34%	29%	19
3	34%	32%	20
4	19%	14%	10

Table 2 : distribution of samples from the 4 genetic categories in the two subgroups KM1 and KM2.

ANNEX III
Individual Progress Report
Participant n° 1

4 Discussion: 4.2 Genetic analyses

PCR results and the hybridization experiment demonstrated that two genomic sequences of the amylases were amplified using the primers AMYH0/HAMY2. These amplified products differ by a length of about 500bp, suggesting that two different amylase genes exist at least. This will be verified in collaboration with P.Boudry (partner 1) and Dr.Kotoulas (partner 5) by looking at parental amylase patterns and their inheritance in the progeny, and using in situ hybridization on chromosomes (Collaboration with G.Rafferty (partner 4) and Dr. C.Thiriot *et al.*(partner 3)).
The PCR using primers AMYH4/HAMY1 revealed that DNA templates existed in all the samples, even when no band appeared using the first primers. Today two possibilities can explain the results with AMYH0/HAMY2 primers: one is that a large intron exists between these primers in some amylase genes, and the polymerase cannot amplify over than 2500bp. Another possibility is that mutations occured at the sites of at least one of the primers. This last hypothesis could also explain the changes in intensity of the 2500bp product.
It seems that differences mainly affect this 2500 pb gene in groups 1, 2 and 3, as in group 4, the two genes are modified with regard to the primers used. The sequence of these genes is under study, and mutations are shown in the coding sequence as well as in the intronic sequences (Sellos *et al.* in prep.). It is necessary to go further in the knowledge of these sequences to define new primers allowing the polymorphism study.

However, the characterization of four groups of samples using the first primer set, led to promising results :

- Samples demonstrating two bands had a significant higher amylase Km (and possibly laminarinase Km) than samples demonstrating one or no bands. These results show that changes in Michaelis constants of amylase protein are related to changes in genetic structure of amylase gene. A better characterization of these changes in the exonic sequence is necessary to corroborate this interpretation.

- It is possible to observe a significant higher RGR between either one of the first 2 categories and the third one. And a good trend in the relationship between these 4 genetic patterns and relative growth rate is displayed when four data out of a normal distribution are not considered . Samples with two bands would demonstrate a higher growth rate than the others. But it is necessary to improve this result and to explain why four data are not in the same trend.

- Subgroup KM1 had 25% more samples from the category 4, and subgroup KM2, twice as many samples from category 1.

The combination of these three results underlines the concomitant higher amylase Michaelis constant and higher relative growth rate in the samples demonstrating two bands. These are more frequent in subgroup KM2. In this subgroup we found also that RGR increased with amylase Km values which corroborates the fact that higher Km values are encountered simultaneously with higher RGR.

These two subgroups defined according to their absorption efficiency characteristics are made up of a similar mixture of the different genetic patterns with few differences in two extremes reflected by PCR with 2 bands and PCR with no bands. This is reflected also in the scattering of enzymatic Km data, where the highest and the lowest values are shown in amylase Km of subgroup KM2.

Even if all the results are converging, none of our criteria are enough alone to explain the two physiological constants KM1 and KM2.

3 Results: 3.3 Digestive enzyme activities and the two subgroups KM1 and KM2:

3.3.1 Relation with ingestion, absorption efficiency and relative growth rate

As previously reported in the last report, the two subgroups reacted to ingestion in opposite ways:

- Digestive levels decreased significantly (p 0.05) in the KM1 subgroup when food ingestion increased. Inversely, enzymatic levels tended to increase in the KM2 subgroup when food increased (Figure 6).
- Digestive enzyme activities were significantly positively correlated with absorption efficiency (p 0.05) in subgroup KM2 and not in subgroup KM1 (Figure.6).
- The two subgroups (Figure 6) demonstrated a different set of relationships between digestive activities, relative growth rate, and absorption efficiency, even if correlations were not statistically significant.

4 Discussion: 4.3 Digestive enzyme activities and the two subgroups KM1 and KM2

In the subgroup with the smallest physiological constant KM1, enzymatic levels decreased when ingestion rate increased. This could be a compensatory mechanism when maximum absorption efficiency is obtained. According to the proposed model :

$$ABS/ING = ABS/ING\ Max^{*}\ (E)(ING)/(KM + ING).$$

This maximum absorption efficiency is obtained at low ingestion rate because KM1 is small. As a consequence, relative growth rate is limited by the small quantities of ingested food. It can be observed that in subgroup KM1, most of the samples have a relative growth rate around the maximum enzymatic values (Figure 6). Few higher RGR were observed at lower enzymatic levels.

In contrast, in the subgroup with a high physiological constant, KM2, the maximum absorption efficiency is obtained only when ingested food is high. As a consequence, absorption efficiency was positively correlated with digestive enzyme activity and ingestion. Relative growth rate will be higher than in the first subgroup, because ingested food will be high when absorption efficiency is at a maximum. Samples with higher relative growth rates are more numerous in subgroup KM2 than in subgroup

KM1, but the means (respectively 0.74 and 0.69) were not statistically different (T test p=0.37, 97 report). (Figure 6).

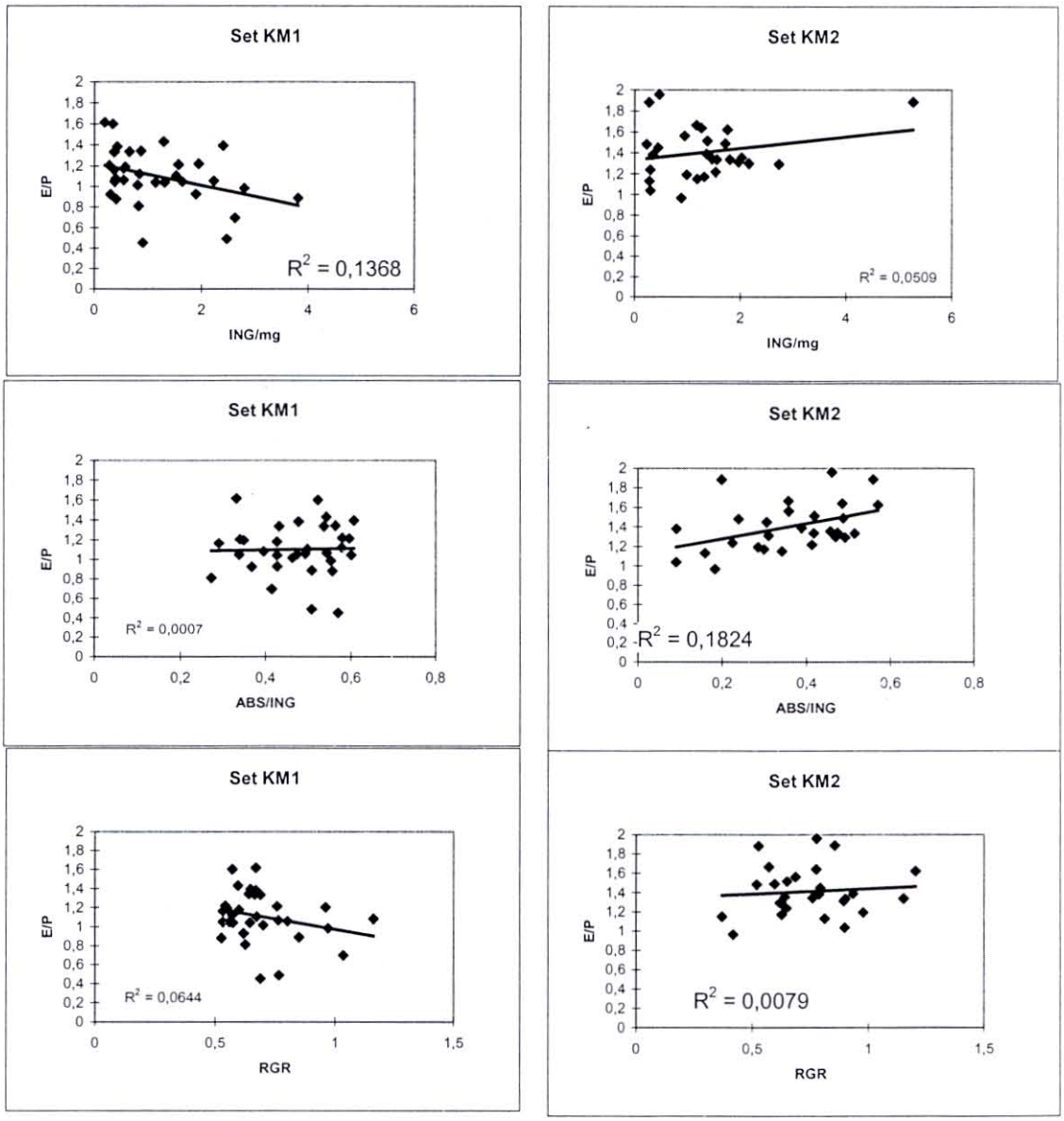


Figure 6: Relationships between specific activity of digestive enzymes (amylase + laminarinase) and ingestion/mg, absorption efficiency and relative growth rate (RGR) in the subgroup KM1 (left) and KM2 (right)

4 Discussion: 4.4 General Discussion

The data obtained today add promising results to our knowledge, it is the first time with molluscs, that it has been possible to show convergence between independant data from a physiological approach (measurement of absorption efficiency), an enzymatical approach (digestive enzymes activities, digestive enzyme Michaelis constants), and a genetic approach (different genetic patterns of the amylase gene).

The proposed model can summarize a part of this convergence, as absorption efficiency depends on enzymatic activities (E) and Ingestion rate (ING), with a saturation process when ingestion rate exceeds the Michaelis constant Km. These catalytic constants are related to a genetic structure.
The two different physiological KM constants shown in the results are not completely determined by the true Michaelis constants of the digestive enzymes, but again, the results demonstrated that Michaelis constants varied with absorption efficiency and growth rate mainly in the KM2 subgroup. This result supports the inclusion of apparent affinity Km of the digestive enzymes in the model. It is probably the case for the physiological constants.

Some correlations are statistically significant, others are trends. In such an approach using only two digestive activities, and only the genetic pattern of one enzyme, one cannot expect strong relationships describing absorption efficiency and growth. The significant correlations observed using this limited number of parameters probably suggest a very strong phenomenon.

These results should be improved by other experiments planned for the future work plan in the three fields :

- Improvement of physiological data and the division of two subgroups with different physiological KM constants
- Improvement of catalytic parameter measurements on other samples .
- Improvement of polymorphism methods : at the genetic level, we have to develop the polymorphism study of amylase genes to better recognize oysters of the two categories
- Characterization of expression (in relation to cellular localization and trophic conditions) and of catalytic properties of proteins from the different genes and alleles.

However, the proposed model can help for the design of future experiments :

- It shows that subgroup KM1 would be adapted to low food concentration, will have a good absorption efficiency in these conditions, but a limited growth rate because ingested food is not very high. When ingested food is at a high level, the decrease in the digestive activities could also limit the increase of absorption efficiency and therefore the increase in growth rate. These animals should be perform continuously in limited trophic conditions. They can be found among animals showing one or no bands using the set of primers described above.

- In subgroup KM2, oysters would be adapted to high trophic situations. They will perform slowly in low trophic situations due to a low absorption efficiency, but when

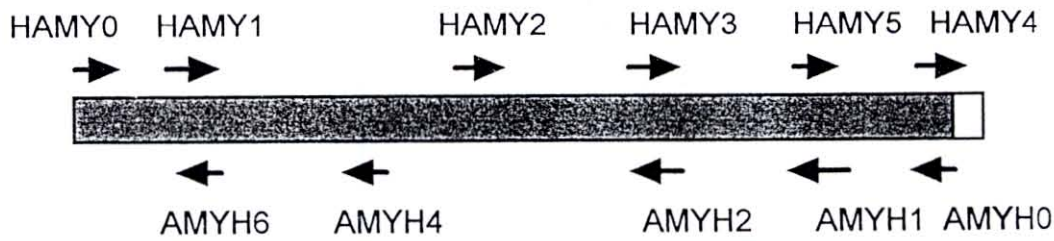
trophic conditions are very good, they should grow very fast over a short period of time because of the high level of food ingested and the high absorption efficiency in these conditions. They can be found among animals showing two bands using the set of primers described above.

Next year, the first priority will be on amylase polymorphism study, related amylase properties and growth performances, on different set of samples : G0 samples for inheritance of polymorphism, G1 Thau samples , for the effect of ecosystem on enzymatic characteristics and G2 selected samples for links with physiological performances.

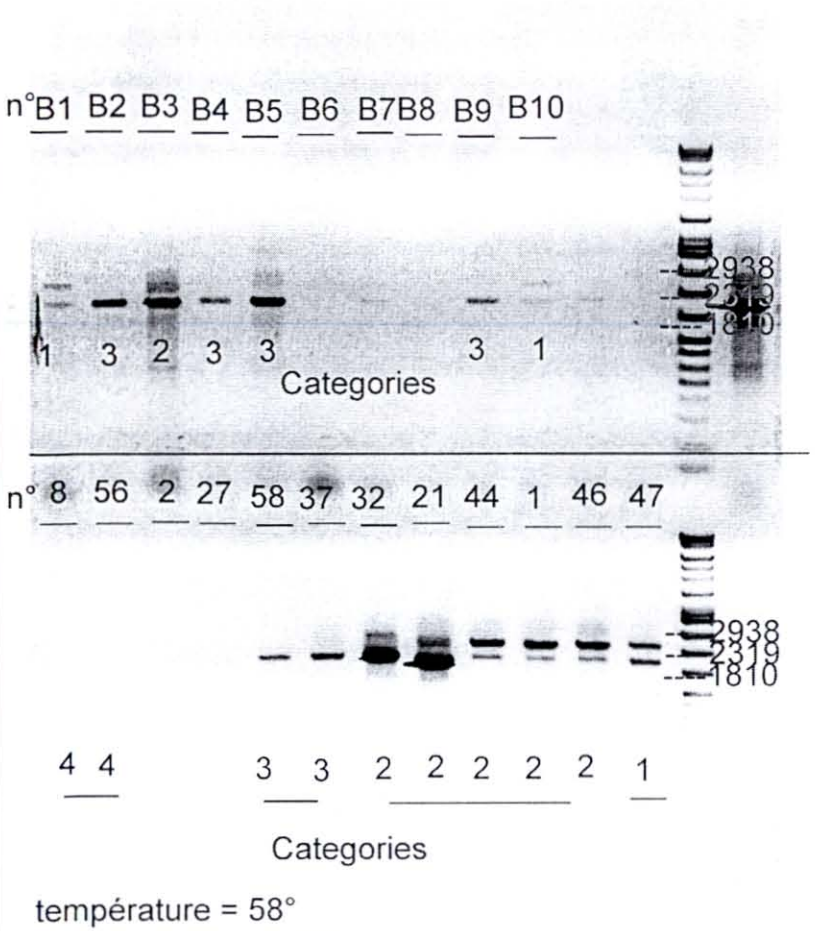
Significant difficulties or delay experienced during the third reporting period

Experiments on larvae previously planned in the project, were delayed to better analyse and develop the 1997 results. In place, a molecular and enzymological approach was developed as annonced in the 1997 report.

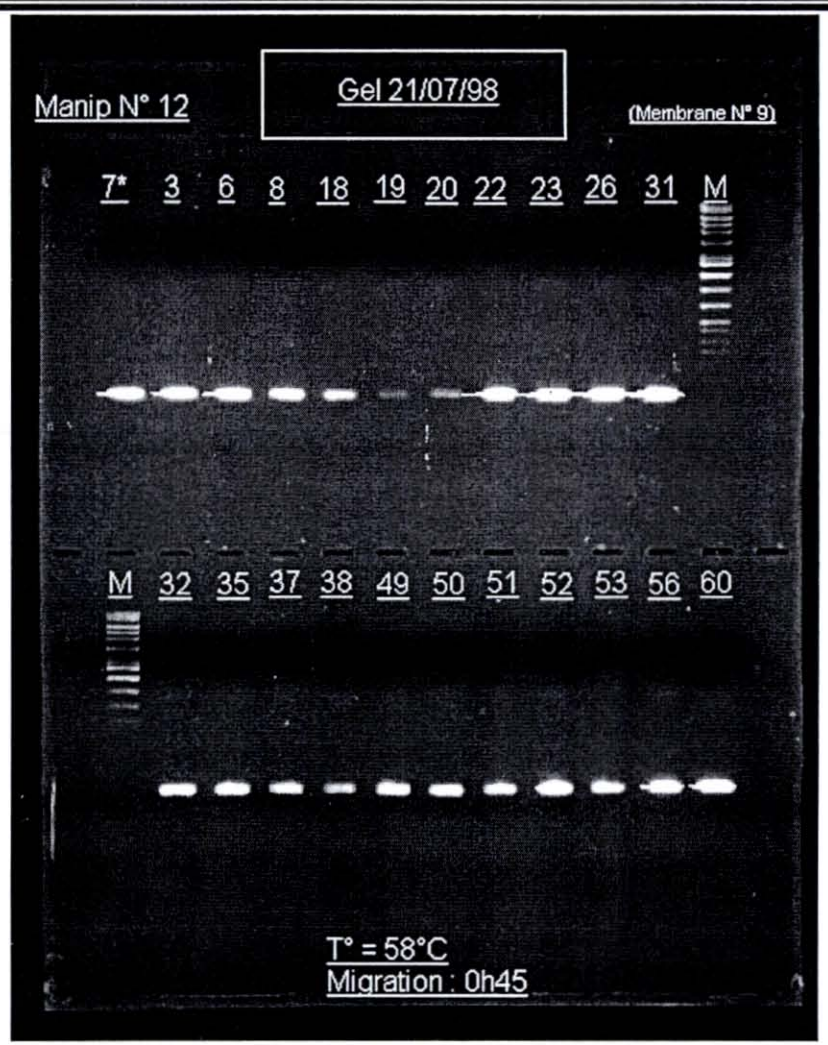
Annexes:



Annex 1 Sites of primers defined on amylase cDNA from the oyster C.gigas. (Scheme provided by Sellos et al).



Annex 2. Photograph of PCR amplified bands from genomic samples PCR on genomic DNA (N° of sample) using AMYH0/HAMY2 primers. Categories are 1 = 2 bands, 2 = 1 major and one minor, 3 = 1 band, 4 = no bands



Control PCR on genomic DNA (N° of sample) using AMYH4/HAMY1 primers. All the samples have the expected band, even those that had no bands using AMYH0/HAMY2 primers, such as N° 8 or 56

KM1			KM2		
Tube n°	Origin	IND	Tube n°	Origin	IND
57	PDB	06h3	9	PDB	05c9
5	ARC	07g1	56	PDB	05f6
64	BON	15d5	43	ARC	09e5
25	PDB	05a3	41	ARC	09g1
27	PDB	06a6	44	ARC	09g6
7	ARC	09b8	19	BON	13f8
48	ARC	16a8	51	PDB	06c6
18	BON	13b4	55	PDB	05d4
60	BON	15a2	14	PDB	05g1
24	PDB	05a8	10	PDB	06b8
53	PDB	05g2	46	ARC	08f6
54	PDB	06h9	16	BON	13e3
6	ARC	09c2	36	BON	14g2
29	ARC	09e6	26	PDB	05b6
61	BON	14b2	47	ARC	08f8
34	BON	14g6	8	ARC	17h1
23	PDB	06h4	67	BON	15a6
12	PDB	06h6	63	BON	15b3
22	PDB	05f9	15	BON	15g8
50	PDB	05h2	39	BON	13e9
13	PDB	17g2	42	ARC	08g3
40	BON	15h6	28	ARC	07h5
37	BON	14f1	32	ARC	07f6
35	BON	15c6	45	ARC	08c6
17	BON	15g5	30	ARC	08d1
68	BON	15b9	49	ARC	09a3
59	BON	14f4	20	BON	13g8
1	ARC	08e2	31	ARC	08c8
3	ARC	09c4			
21	PDB	04h8			
2	ARC	09f6			
65	BON	14b4			
33	ARC	07b7			

Annex 3: Data Set, KM1 and KM2 Physiological subgroups.

Tube n°	IND	Gen. Cat.1	Tube n°	IND	Gen. Cat.2	Tube n°	IND	Gen. Cat3	Tube n°	IND	Gen. Cat.4
31	08c8	1	33	07b7	2	3	09c4	3	28	07h5	4
1	08e2	1	32	07f6	2	29	09e6	3	2	09f6	4
46	08f6	1	5	07g1	2	39	13e9	3	41	09g1	4
47	08f8	1	45	08c6	2	19	13f8	3	8	17h1	4
6	09c2	1	30	08d1	2	20	13g8	3	60	15a2	4
43	09e5	1	42	08g3	2	37	14f1	3	17	15g5	4
44	09g6	1	49	09a3	2	59	14f4	3	24	05a8	4
48	16a8	1	7	09b8	2	34	14g6	3	56	05f6	4
36	14g2	1	18	13b4	2	68	15b9	3	27	06a6	4
67	15a6	1	16	13e3	2	15	15g8	3	12	06h6	4
35	15c6	1	65	14b4	2	26	05b6	3			
			63	15b3	2	9	05c9	3			
			64	15d5	2	22	05f9	3			
			40	15h6	2	14	05g1	3			
			21	04h8	2	53	05g2	3			
			25	05a3	2	10	06b8	3			
			55	05d4	2	51	06c6	3			
			50	05h2	2	57	06h3	3			
			54	06h9	2	23	06h4	3			
						13	17g2	3			

Annex 3 Data set: Genetic (amylase) categories 1: 2 bands same amplification, 2 : 2 bands one low amplification., 3 : 1 band, 4 : no bands

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 1998

Partner n°2

NERC
Natural Environment Research Council

Plymouth Marine Laboratory

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual progress report
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 1998**

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

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 :
Dr Serge BOUGRIER
(IFREMER L'Houmeau)

Participant n°2

Natural Environment Research Council

Plymouth Marine Laboratory
Centre for Coastal and Marine Sciences
Prospect Place
Plymouth PL1 3DH
United Kingdom

Ass. Contractor to the coordinator — Dr A J S Hawkins (Contractor)

ANNEX III
Individual progress report
Participant n°2

FAIR PL. 95.421
Individual Progress Report
For the period from
1st January to 31st December 1998

Participant n° 2: **Natural Environment Research Council**

Plymouth Marine Laboratory
Centre for Coastal and Marine Sciences

Prospect Place
Plymouth PL1 3DH
United Kingdom

Scientific Team Dr A J S Hawkins
Ms A J Day

Objectives

Recording physiological performances of generations G1 and G2.

Actions in the project

Task 4	Physiological analyses
Sub-task 4.3	Measurement of proteolytic activity and protein turnover
Task 8	Data processing and workup, and report writing
Task 9	Project organisation

Planned research activities

Task	Year 1	Year 2	Year 3	Year 4	Year 5
4.3		Physiological analyses	Physiological analyses	Physiological analyses	Physiological analyses
8		Data workup and report	Data workup and report	Data workup and report	Data workup and final report
9	2 meetings with other participants	1 meeting with other participants	1 meeting with other participants	1 meeting with other participants	1 meeting with other participants

Research activities during the third reporting period

Task 4 Physiological analyses

Sub-task 4.3
Measurement of proteolytic activity and protein turnover

1. Introduction

During the third year of our programme, we have related lysosomal proteolytic activity and whole-body protein synthesis to different rates of growth between individuals from our mixed G1 generation of the oyster *Crassostrea gigas*. Results have been compared with previous findings for the mussel *Mytlilus edulis.* We have also developed an assay for calcium dependent proteases (CDP), that play an important role in intracellular protein degradation (Croall & DeMartino 1991, Pontremoli & Melloni 1986).

2. Methods

2.1 General

Two collaborative experiments were undertaken with scientists at IFREMER La Tremblade, France (Participant n° 1) as follows:

1. June 1997:
   (a) Measuring whole-body protein synthesis in a total of 40 fast- and slow-growing *Crassostrea gigas* from each of three G1 (5 x 5) populations (Arcachon, Port des Barques and Bonne Anse) (Stock B1.2) that had been continuously reared in the laboratory and previously acclimated to 2 mg l^{-1} *S. costatum* at 14.5 °C over 4 weeks, and physiology measured in the same oysters by Participant n° 1 over the preceeding 2 wk; and
   (b) dissecting the above and additional oysters for assays of proteolytic activity.
2. July 1997:
   Samples of digestive gland, gill and adductor muscle from our experiment in June 1997 were lost upon thawing during transfer between laboratories. Therefore, additional samples were collected from Bouin in July 1997(Stock R.1), to ascertain whether any growth-dependent differences in protein metabolism were tissue-specific.

For each experiment, sampled tissues included the digestive gland, gill, adductor muscle and all remaining tissues combined ("the remainder"). All samples were stored at –80 °C. A summary of analyses, tissue divisions and actual sample sizes is given for each experiment in Table 1.

Experiment	Oyster history	Digestive gland	Gill	Adductor muscle	Remaining tissues
June 1997: G1, mixed parentage, from Bonne Anse, Archachon and Port des Barques	Stock B1.2 reared in hatchery	Samples lost	Samples lost	Samples lost	n = 40
July 1997: G1, parents from Bonne Anse only	Stock R.1 reared in nature at Bouin	n = 61	n = 61	n = 61	n =10 "large" oysters n =10 "small" oysters

Table 1: Analyses, tissue divisions and sample sizes

2.2 Measures of whole-body protein synthesis

Rates of protein synthesis were determined *in vivo* by quantitatively administering each mussel ^{15}N-labelled *Isochrysis galbana*, and monitoring the subsequent excretion of ^{15}N as ammonium over 48 h according to an end-product analysis as described by Hawkins et al. (1987).

2.3 Measures of lysosomal proteolytic enzyme activity

Frozen tissue samples were sub-divided for separate analyses of lysosomal enzyme activity, CDP activity, and in the case of gill alone, for stress proteins. Prior to sub-division, the frozen wet weight was obtained for each tissue. For digestive gland and gill, the tissue was broken in liquid nitrogen and fragments weighed for each analysis. Remaining tissues were ground to a fine homogeneous powder in liquid nitrogen, and aliquots of powdered tissue weighed for measures of CDP and lysosomal enzyme activities.

Samples for analyses of lysosomal enzyme activities were homogenised with an ultra-turrax homogeniser in CMFS buffer pH 7.3 in the proportion 1g tissue to 5 ml buffer. 200 µl of the homogenate was frozen immediately for analysis of total protein. The rest of the homogenate was centrifuged at 20,000 g for 30 min and then three 200 µl aliquots of the supernatant were frozen at –80 °C. Supernatant aliquots were all freeze-thawed twice to rupture any intact lysosomes and thus release maximal activity.

Protein in each sample of total homogenate was precipitated by the addition of 20 % perchloric acid, pelleted by centrifugation at 12,000 g and solubilized in 0.5 M NaOH. The solubilized protein was then analysed using the method of Lowry et al. (1951). Protein purified from *C. gigas* adductor muscles (Kreeger & Langdon 1994) was used as a standard.

Cathepsin D was measured in microfuge tubes using 50 µl of 2 % haemoglobin as a substrate, 50 µl of 1 M formate buffer pH 3.6 and 40 µl of sample. The assay was started by the addition of sample and stopped after 20 min by the addition of 3 % TCA. Proteolytic products were visualized using modified alkaline copper and Folin ciocalteau reagents according to Barrett & Heath (1977). Other proteolytic enzymes, particularly cathepsin L, may degrade haemoglobin under these assay conditions. Therefore, for each sample, assays were also ran that included pepstatin A (an inhibitor of cathepsin D) at a concentration of 10 µg ml^{-1}. Cathepsin D activity was obtained by subtracting residual activity remaining after pepstatin inhibition from the total activity. Enzyme units are expressed as µg tyrosine equivalents per minute.

Cathepsin B was measured using the synthetic substrate Z-Arg-Arg-7AMC (supplier Bachem). Degradation of this substrate releases the fluorescent product, 7AMC. Either 10 µl of digestive gland sample or 20 µl of other tissue samples were pre-incubated for 1 minute in thermally-regulated cuvettes with 400 µl 0.1% Brij and an equal volume of 0.1 M tris-malate buffer, pH 7 containing mM EDTA and mM cysteine. The assay was started by the addition of 200 µl of substrate (concentration 40 µM) and monitored by chart recorder for 2 min in a fluorimeter according to Sarath et al. (1989). Results were compared to 0.5 µM 7AMC and an enzyme unit is equivalent to µmol product per minute.

Leucine aminopeptidase was measured according to Young *et al.* (1979), using L leucine β-napthylamide as a substrate. Either 5 or 10 µl of sample were added to 500 µl 0.1 M tris-malate buffer pH 7.4. The assay was started by the addition of 200 µl substrate producing a concentration in the assay of 6 mM. The reaction was stopped after 4 to 6 min by the addition of 500 µl of fast garnet/SDS. Rates of activity were very high. Therefore, to yield absorbances that were in the linear range for the spectrophotometer, 2.42 ml of distilled water representing a 3 x dilution were added prior to measuring absorbance. One unit of LAP activity was a change of 1.0 absorbance unit min^{-1} in an assay volume of 1.25 ml.

2.4 Developmental work on CDP assay

Adductor muscles from approximately 100 large (> 6cm) *Crassostrea gigas*, obtained from Seasalter Ltd., Whitstable, Kent, were dissected out and frozen in liquid nitrogen. These adductor muscles were separated into two batches and stored at -70°C. Adductors from one batch were used for the preparation of a protein substrate while adductors from the other batch were used to prepare enzyme supernatants to be tested for CDP activity. For the enzyme supernatants, adductors from 15 to 20 oysters were pooled and ground with buffer containing 10 mM Tris, 10 mM NaCL, 0.1mM EDTA and 10 mM dithiothreitol. Tissues were homogenised using an ultra-turrax homogeniser in the proportion 1 g to either 5 or 10 ml of buffer, and centrifuged at 20,000 g for 60 min. Supernatants were frozen in 1 ml aliquots prior to assay trials.

Activity of CDP *in vivo* is regulated by calpastatin. The presence of this inhibitor in enzyme supernatants prevents the detection of CDP activity. It is therefore necessary to remove this inhibitor by ion-exchange chromatography prior to enzyme analysis (Waxman, 1981). Our purification proceedure followed the scaled-down method for small samples of Tawa *et. al.* (1992). Ion-exchange columns with a bed volume of 1 ml and containing weak anion-exchange media were obtained from Whatman (mini express column D). The columns were pre-equilibrated with homogenising buffer following manufacturers recommended proceedures. One or two mls of enzyme supernatant were loaded onto each column, which was then washed with more homogenising buffer to remove unbound material. The columns were then eluted with up to ten 1 ml aliquots of homogenising buffer that contained between 0.05 M to 0.5 M sodium chloride, at concentrations which increased with each added aliquot. In mammals, calpastatin elutes at a salt concentration of 0.15 M and CDP at a concentration of 0.3 M. However, it could not be assumed that elution conditions would be similar for oysters. Therefore, salt concentrations in elution buffers varied between trials. In all cases, for each 1 ml of elution buffer, a 1 ml eluate fraction was collected. Each eluate fraction was frozen at -70°C and subsequently tested for CDP activity.

The assay for CDP activity was carried out in Eppendorf tubes. 150 µl of each eluate fraction were pre-incubated for 15 mins with either 50 µl of 5 mM calcium chloride or 5 mM EDTA. The EDTA incubation acts as a control to measure non-CDP activity. Following the pre-incubation, 500 µl of substrate reagent were added. The substrate reagent contained 200 µl of substrate and 300 µl of 0.2 M tris-Cl buffer with 10 mM Cysteine. The pH of the assay buffer varied from 6.1 to 9.0 resulting in an assay pH that varied from 6.5 to 8.4. Assay incubation times varied from 0.5 h to 17 h with the assay being stopped by the addition of 750 µl of 20 % TCA. Assay temperature was either 20 °C for the longer incubations or 25 °C for shorter incubation times. All assay tubes were then centrifuged and the absorbance of the supernatant measured at 280 nm. Net absorbance due to CDP activity was obtained by subtraction of the absorbance from the assay with EDTA from that with calcium.

Two different substrates were used in our trials. These included N' N' dimethylated casein obtained from Sigma and protein purified from *C. gigas* adductor muscles using the method of Kreeger and Langdon (1994). Both substrates were alkali-denatured according to the method of Ashby and Walsh (1974). Following denaturing, the pH was adjusted to 7.0 and the concentration adjusted to 30 mg ml^{-1}.

3. Results

3.1 Measures of lifetime growth and whole-body protein synthesis

Integrated lifetime growth was measured as size, given that all oysters were bred from the same cohort.

Figure 1 illustrates a strong positive relation between growth measured as the total wet weight of both shell and soft tissue and growth measured as the dry weight of soft tissues alone ($p < 0.000001$). Within that relation, there was no difference between the offspring from parents that had been collected from different source populations ($p > 0.05$) (figure 1).

Figure 2 illustrates strong negative allometric relations between weight corrected rates of whole-body protein synthesis and integrated lifetime measured both as the total wet weight of shell and soft tissue and the dry weight of soft tissues alone. Similarly, there were strong negative allometric relations between weight corrected rates of net ammonia loss and growth measured both as the total wet weight of shell and soft tissue and the dry weight of soft tissue alone (figure 2).

Given that these rates of protein synthesis and ammonia loss had been standardised for differences in body size, above relations establish that protein turnover and nitrogen loss were faster per unit tissue of the slower-growing oysters.

3.2 Measures of lysosomal proteolytic enzyme activity

3.2.1 Species comparison

Figure 3 illustrates how specific activities of cathepsin B were similar for each tissue division within both the oyster *Crassostrea gigas* and the mussel *Mytilus edulis*. However, specific activities for cathepsin D and leucine aminopepdidase were over ten times higher in *C. gigas* (figure 3), although in *M. edulis* used the same substrates as *C. gigas*, and were optimised to yield maximal activity.

3.2.2 June 1997: Crassostrea gigas

There were strong positive linear relations between the weight-corrected activities of each studied enzyme within "remaining tissues", especially between leucine aminopepdidase and cathepsin D ($p = 0.0000005$).

Figure 4 illustrates strong negative allometric relations between the weight-corrected acitivities of each studied enzyme within "remaining tissues" and whole-animal growth measured both as the total wet weight of shell and tissues and as the dry weight of all soft tissues alone ($p < 0.003$).
Figure 5 illustrates how the weight-corrected acitivity of leucine aminopepdidase within "remaining tissues" varied in strong positive allometric relation with weight-corrected rates of whole-body protein synthesis ($p = 0.002$), including how the weight-corrected acitivities of each studied enzyme varied in strong positive allometric relations with net ammonia loss ($p < 0.03$). Neither of the relations depicted between whole-body protein synthesis and the activities of cathepsin B ($p = 0.24$) or cathepsin D ($p = 0.06$) were statistically significant.

3.2.3 July 1997: Crassostrea gigas

Figure 6 illustrates strong negative allometric relations between oyster size/growth and the weight-corrected activities of each studied enzyme within all soft tissues combined ($p < 0.001$). This establishes that similar relations that we observed for "remaining tissues" alone in June 1997 also occur within the whole animal.

To analyse whether these differences in whole-body enzyme activity were tissue-specific, we compared the relative contributions of gill, digestive gland and remaining tissues to whole-body enzyme activities, dividing these same 20 oysters into fast- and slow-growing classes according to whether their whole-body soft tissues weighed more or less than 2 g. Figure 7 illustrates how the digestive gland may have been marginally ($p = 0.05$) smaller in faster-growers than in slower-growers. But this was a small difference, with basic similarity in the relative proportions that each tissue division contributed to the whole-body soft tissue weight. Alternatively, as is illustrated in figures 8 and 9, there were no differences between fast- and slow-growers in the average weight-corrected activity of cathepsin B within any of the three tissue divisions ($p > 0.05$), but the average weight-corrected activity of cathepsin D was significantly higher in both the digestive gland ($p = 0.04$) and the remaining tissues ($p = 0.03$) of slower-growers. Further, the average weight-corrected activity of leucine aminopepdidase was significantly higher in the remaining tissues of slower-growers ($p = 0.05$), and there was a suggestion of higher associated activity in the digestive gland, but which was not statistically significant (figure 10).

3.3 Developmental work on CDP assay

To compare substrates, eight elution buffers that varied between 0.05 M and 0.4 M salt were used to generate eight eluate fractions.The pH optima for CDP's in other species of molluscs and mammals has been shown to vary between 6.8 and 7.4. Therefore, activity was measured initially at pH 7.4. To maximise activity, incubations were carried out over 17 hours at a temperature of 20°C.
Net activities in each eluate fraction are plotted in figure 11a for casein and figure 11b for muscle protein. Results were conflicting. Although substrates were tested on the same eluate fractions, net CDP activity was observed in the 0.2 M fraction with casein and the 0.3 M fraction with muscle protein. Muscle protein was selected for use in further trials, because observed activity was more than double that seen with the casein, and was located in the salt fraction which for other species usually eluted the CDP.

To establish pH profiles, two series of pH profiles at 6.5 to 7.8 and 7.4 to 8.4 were measured using the 0.3 M fraction over 17 h at 20 °C. Results are shown in figure 12. CDP activity was seen at pH 7.4 and above, with peak activity at pH 7.8.

The timecourse of CDP activity was measured twice, both times at pH 7.8 on muscle protein. From the first run using time intervals up to 18.5 h, it was apparent that the increase in activity declined rapidly after 2 h (figure 13a). Therefore, for the second run, measurements were made at 15 minute intervals up to 1 3/4 h (figure 13b). No activity

was observed in the first 15 min. However, between 0.5 h and 0.75 h, activity was more or less linear.

To establish the location of CDP activity, eight eluate fractions containing from 0.05 M salt to 0.4 M salt were tested for CDP activity using revised conditions determined from the above experiments. Activity on muscle protein was measured over 1 h at pH 7.8 at 25 °C. CDP activity was detected in all fractions containing more than 0.25 M salt (figure 14). The greatest activities were found in the 0.35 M fraction and the 0.3 M fraction. Activities in the 0.25 M and 0.4 M fractions were at trace levels only.

4. Discussion

Our findings compliment earlier work investigating the metabolic basis of faster growth. Whether following selection or genetic manipulation such as in the induction of triploidy or crossing to induce hybrid vigour, as well as in natural associations with genetic polymorphism measured as heterozygosity, the main consistent basis of genetic increases in growth and other elements of performance are reduced energy requirements and higher growth efficiency, rather than faster feeding and all other associated (Hawkins & Day 1997). Further, it is known that those reduced energy requirements in faster growing individuals stems from decreased intensities of protein metabolism measured at the level of the whole animal (Hawkins & Day ibid, 1998).
In the present programme, slower rates of protein synthesis in faster-growing oysters indicate that a greater proportion of that synthesis must have been effecting net protein deposition than in slower-growing oysters, so that the intensity with which proteins were being replaced and renewed (= protein turnover) was greater in those slower-growers. This suggests, as in past studies, that slower growth in the first GI generation of this GENEPHYS programme resulted at least in part from the high energetic costs of protein turnover, together with faster rates of nitrogen excretion.

Activities of both leucine aminopepdidase and cathepsin D were higher in the remaining tissues of slower-growers, confirming that the faster whole-body protein synthesis in slower-growers stem from fundamental metabolic differences in non-digestive tissues, and were not directly related to feeding rate. These findings also establish that differences in whole-body protein turnover and growth efficiency stem from extracellular proteolytic activity, for both leucine aminopepdidase and cathepsin D are located mainly in lysosomes.

To further understand these interrelations, we have now completed development of a novel assay for cytosolic calcium-dependent proteases, which we will shortly apply in the above samples from both June and July 1997. This will tell us whether differences in whole-body protein turnover derive solely from extracellular lysosomal activity, or whether they also stem from associated differences in basal intracellular metabolism.

Whilst developing of our assay for calcium-dependent proteases, we have found calcium-dependent activity in the adductor muscle of *C. gigas*, which degraded proteins from this same tissue within a pH range of 7.4 to 8.4 and optimally at pH 7.8. This pH optimum is higher than those reported for other molluscs (pH 6.8 in scallop, Maeda et al. 1992; pH 6.8 in *Mytilus edulis* Day & Hawkins unpublished; pH 7.0 in octopus, Hatzizisis et al. 1996) and mammals (for example, pH 7.4 in the rat, Tawa et al. 1992). Optimal elution conditions also differ from those in other molluscs. Calcium-dependent proteases from *Crassostrea gigas* elute at a salt concentration above 0.25 M and optimally between 0.3 M and 0.35 M. This differs markedly from the 0.2 M salt concentration at calcium-dependent proteases elute in octopus (Hatzizisis et al. 1996). These collective differences are perhaps not surprising given the diversity of both structure and biochemical properties that have been reported for calcium-dependent proteases among invertebrates (Beyette et al. 1993). For example, calcium-dependent proteases vary in size from 59 kDa in lobster (Mykles & Skinner 1986) to 850 kDa in scallop (Maeda et al. 1992).

5. References

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ANNEX III
Individual progress report
Participant n°2

Task 8 Data processing and workup, and report writing

Data processing and workup is progressing well. When we have completed our analyses of calcium-dependent proteases in samples from June and July 1997, we will be in a good position to effect a fully integrated workup combining the genetic data that is available from Participant no 4 and the physiological data of Participant no 1. This Report was posted to our project co-ordinator on 21 December 1998.

Task 9 Project organisation

Project organisation was facilitated through the collective meeting of principle investigators from most Participants in Galway, Ireland, from 2-4 November 1998. This was a successful meeting that enabled an enthusiatic consolidation and planning.

Significant difficulties or delays experienced during the third reporting period

There have been no significant delays or difficulties experienced during this reporting period. The development of our assay to measure the cytosolic proteolytic activity of calcium-dependent proteases has taken longer than expected, as optimal assay conditions are so different from those in higher animals. But that development is now complete, and has yielded novel findings that are of added value.

Figure 1. Crassostrea gigas. Relation between lifetime growth measured as the total weight of both shell and wet tissue and lifetime growth measured as the dry weight of soft tissues alone. Data are from the GENEPHYS G1 generation (Stock B1.2) that had been reared within the La Tremblade hatchery. Parentage is designated according to oysters from Arcachon (A), Port des Barques (P) or Bonne Anse (B).

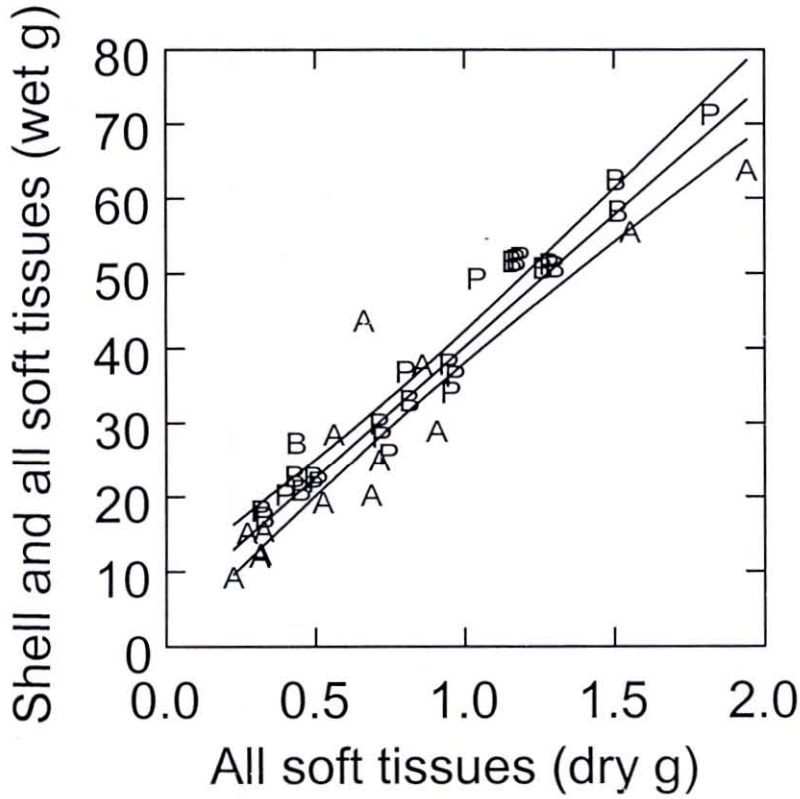


Figure 2. Crassostrea gigas. Relations between rates either of (a) whole-body protein synthesis (mg protein d^{-1}) or of (b) net ammonia loss (µmol h^{-1}) and integrated lifetime growth measured both as total wet weight of shell and soft tissues and the weight of dry soft tissues alone. Data are from the GENEPHYS G1 generation (Stock B1.2) that had been reared within the La Tremblade hatchery, and are weight-corrected for a standard oyster of 1 g soft dry tissue weight. Parentage is designated according to oysters from Arcachon (A), Port des Barques (P) or Bonne Anse (B)

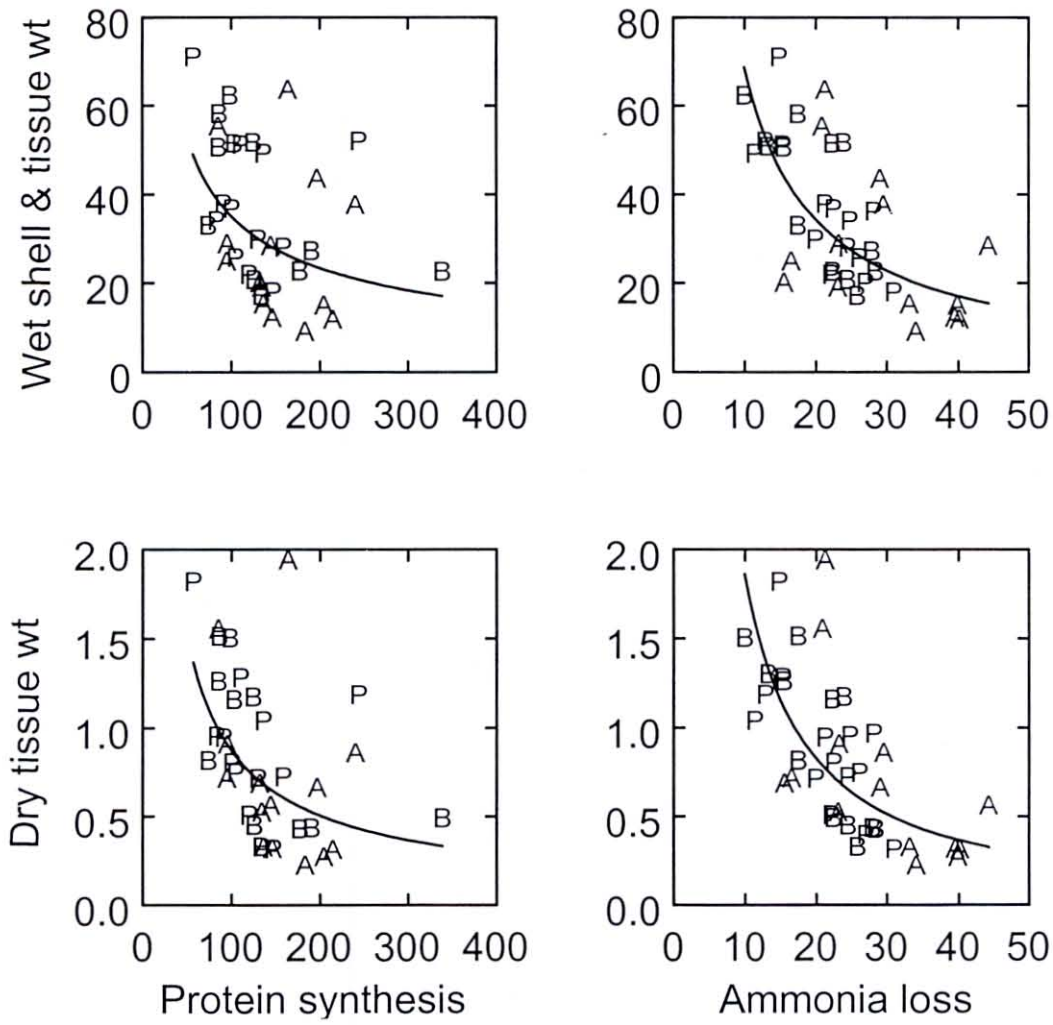
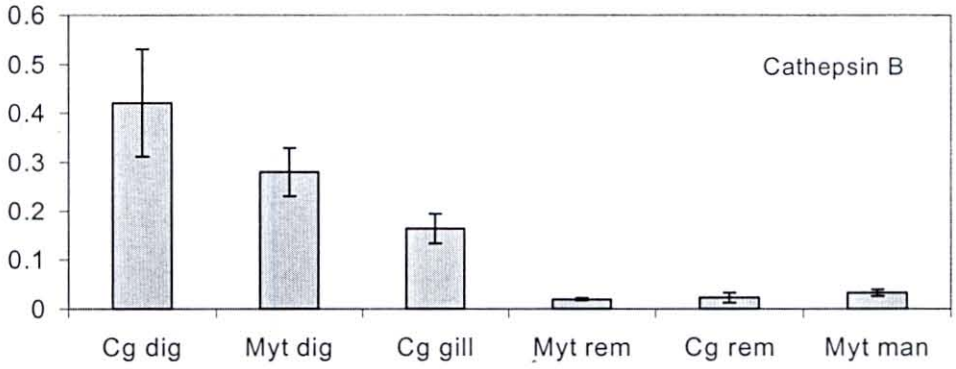
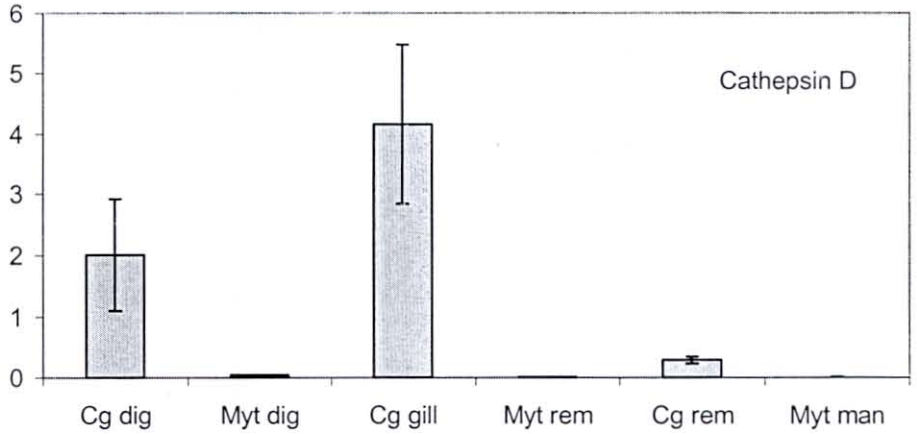


Figure 3. Comparsion of the specific activities of cathepsin B (nmol min^{-1}), cathepsin D (µg min^{-1}) and leucine aminopepdidase (units min^{-1}) within the digestive gland (dig), gill and all remaining tissues (rem) of the oyster Crassostrea gigas (Cg) and the mussel Mytilus edulis (Myt).





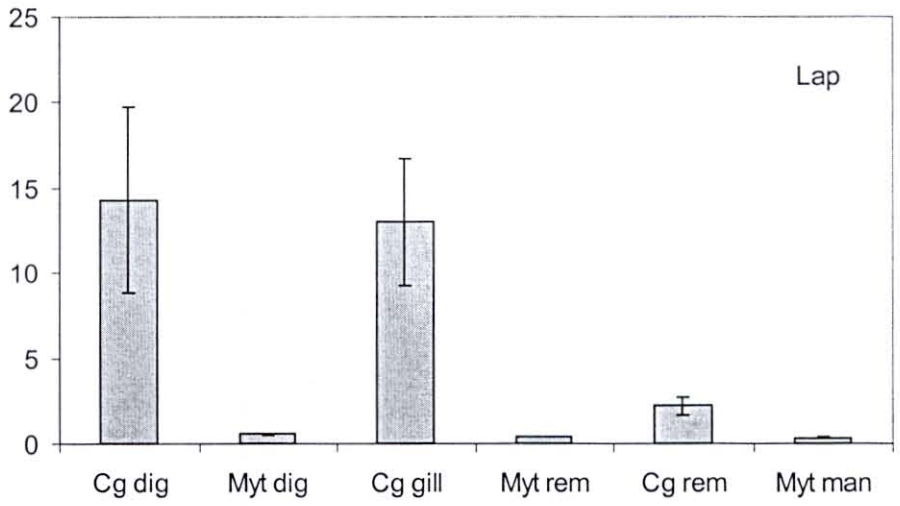


Figure 4. Crassostrea gigas. Relations between the specific activities of cathepsin B (CB) (nmol min^{-1}), cathepsin D (CD) (µg min^{-1}) or leucine aminopepdidase (LAP) (units min^{-1}) within "remaining tissues" and integrated lifetime growth measured both as the total wet weight of shell and soft tissues and the dry weight of soft tissues alone. Data are from the GENEPHYS G1 generation that had been reared within the La Tremblade hatchery (Stock B1.2), and are weight-corrected for a standard oyster of 25 mg soft tissue protein Parentage is designated according to oysters from Arcachon (A), Port des Barques (P) or Bonne Anse (B).

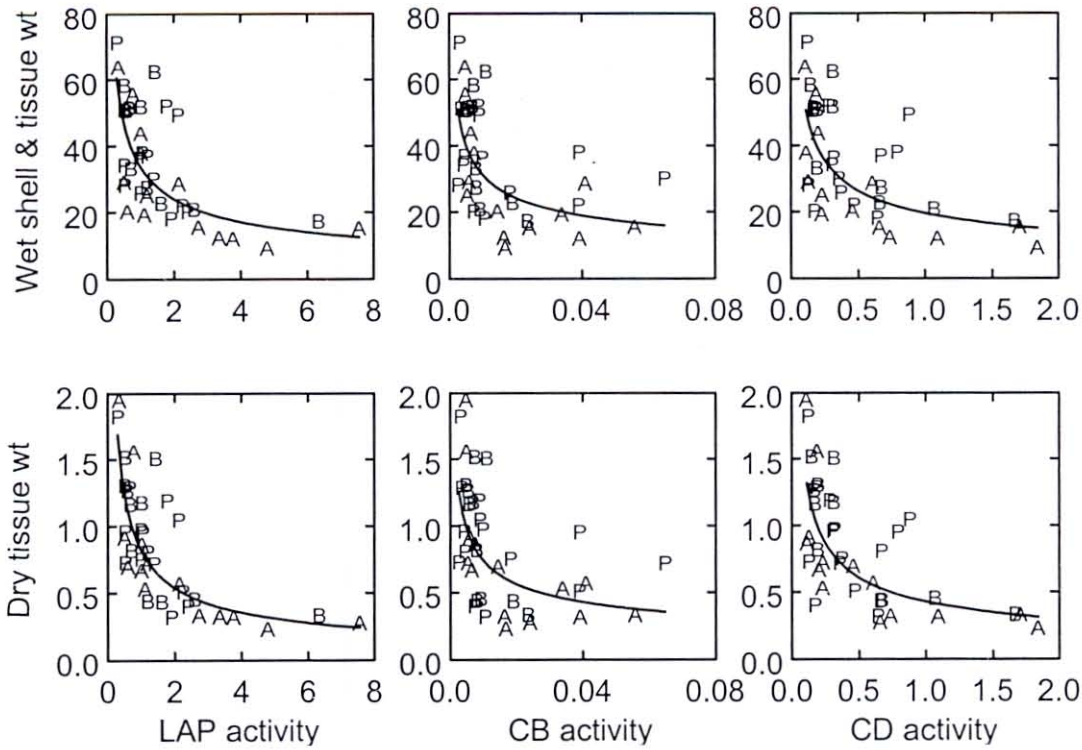
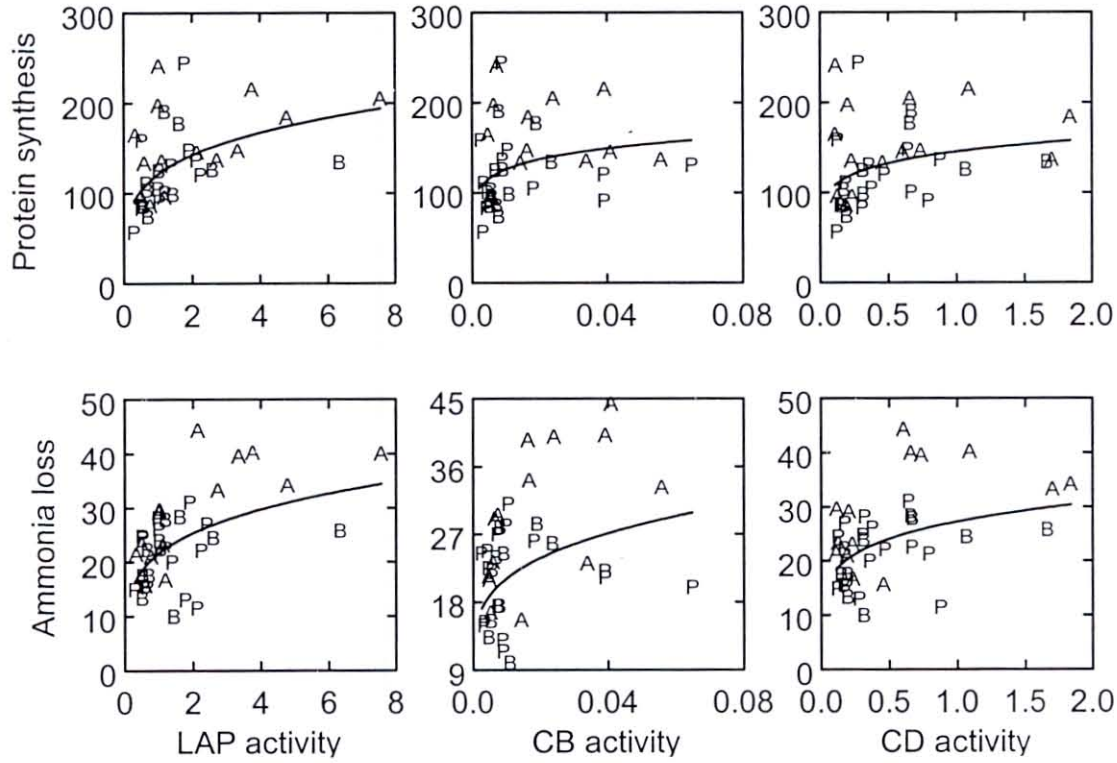


Figure 5. Crassostrea gigas. Relations between the specific activities of cathepsin B (CB) (nmol per 25 mg protein min^{-1}), cathepsin D (CD) (µg per 25 mg protein min^{-1}) or leucine aminopepdidase (LAP) (units per 25 mg protein min^{-1}) within "remaining tissues" and rates either of whole-body protein synthesis (mg protein g^{-1} soft dry tissue d^{-1}) or of net ammonia loss (µmol g^{-1} soft dry tissue h^{-1}). Data are from the GENEPHYS G1 generation (Stock B1.2) that had been reared within the La Tremblade hatchery. Parentage is designated according to oysters from Arcachon (A), Port des Barques (P) or Bonne Anse (B).



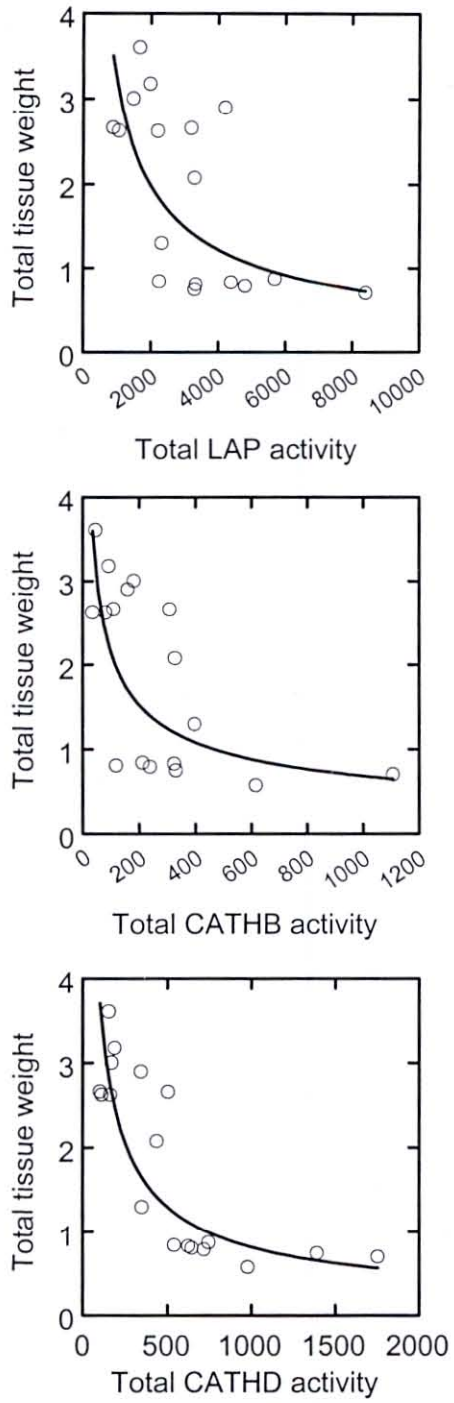


Figure 6. Crassostrea gigas. Relations between the specific activities of cathepsin B (CB) (nmol min^{-1}), cathepsin D (CD) (µg min^{-1}) or leucine aminopepdidase (LAP) (units min^{-1}) within all soft tissues combined and integrated lifetime growth measured as the dry weight of all soft tissues. Data are from the GENEPHYS G1 generation that had been reared in the natural environment at Bouin (Stock R.1). Enzyme activities are weight-corrected for a standard oyster of 25 mg soft tissue protein. Each oyster was bred from parents from Bonne Anse alone.

Figure 7. Crassostrea gigas . Contributions of digestive gland, gill and all remaining tissues (remainder) towards the dry weight of all soft tissues combined in slow- (< 2 g dry soft tissue) and fast-growers (> 2 g dry soft tissue) from the GENEPHYS G1 generation that had been reared in the natural environment at Bouin (Stock R.1). Data are the means ± 2 SE (n = 20). Each oyster was bred from parents from Bonne Anse alone.

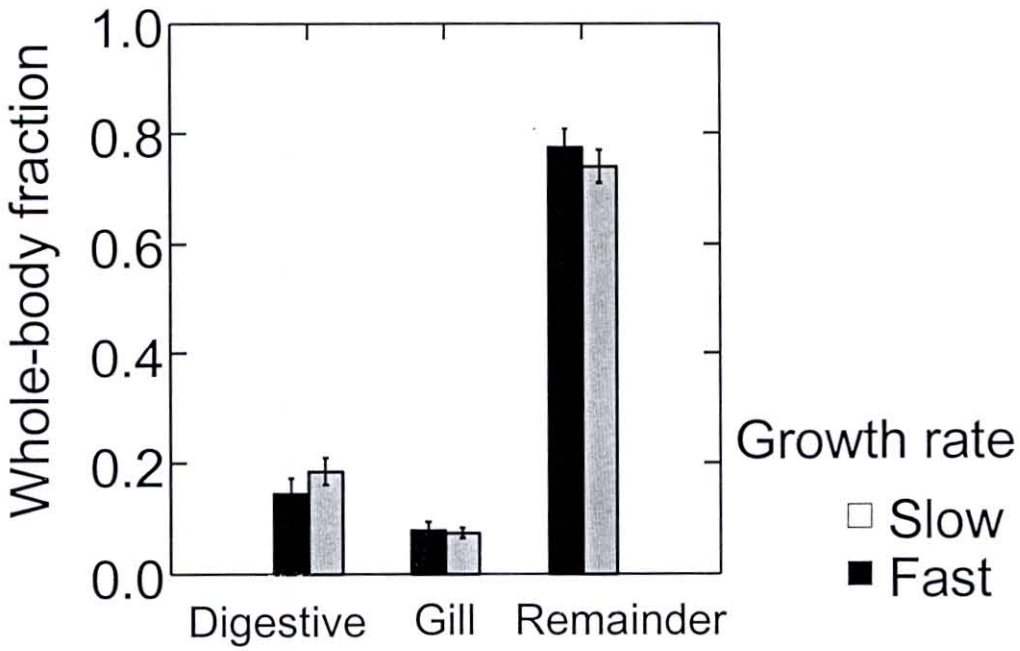


Figure 8. Crassostrea gigas . Specific activities of cathepsin B (nmol min^{-1}) within the digestive gland, gill and all remaining tissues (remainder) of slow- (< 2 g dry soft tissue) and fast-growers (> 2 g dry soft tissue) from the GENEPHYS G1 generation that had been reared in the natural environment at Bouin (Stock R.1). Data are the means ± 2 SE (n = 20). Enzyme activities are weight-corrected for a standard oyster of 25 mg soft tissue protein. Each oyster was bred from parents from Bonne Anse alone.

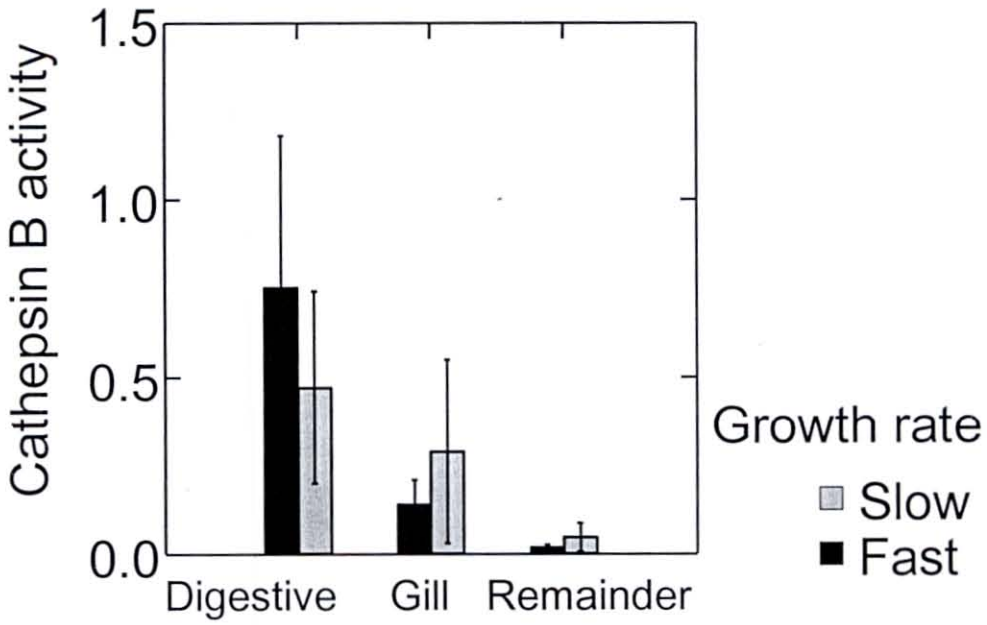


Figure 9. Crassostrea gigas . Specific activities of cathepsin D (µg min^{-1}) within the digestive gland, gill and all remaining tissues (remainder) of slow- (< 2 g dry soft tissue) and fast-growers (> 2 g dry soft tissue) from the GENEPHYS G1 generation that had been reared in the natural environment at Bouin (Stock R.1). Data are the means ± 2 SE (n = 20). Enzyme activities are weight-corrected for a standard oyster of 25 mg soft tissue protein. Each oyster was bred from parents from Bonne Anse alone.

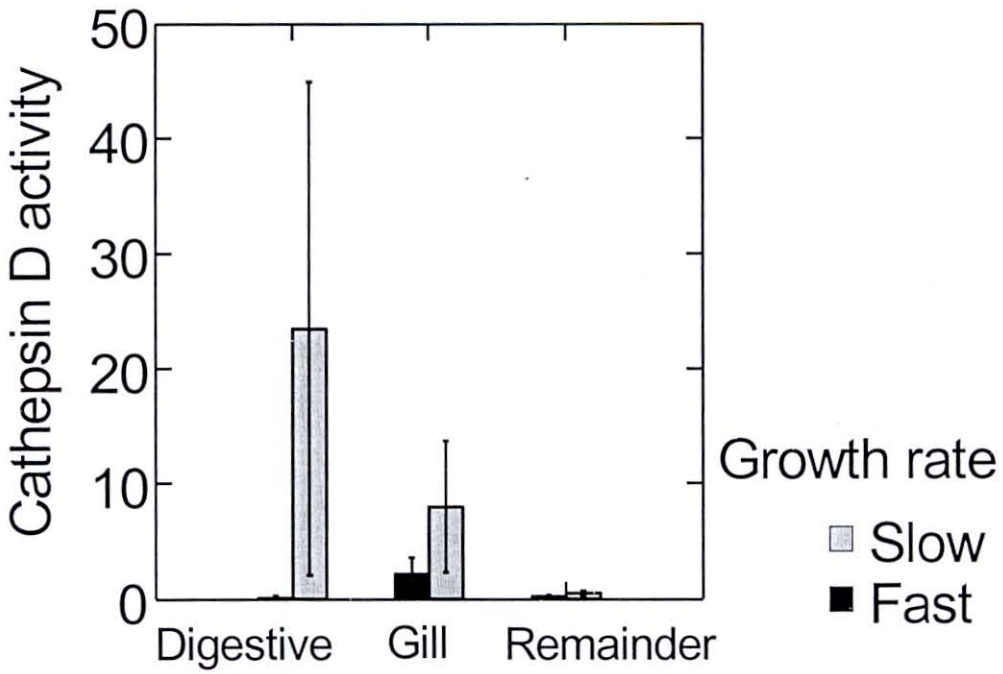


Figure 10. Crassostrea gigas. Specific activities of leucine aminopepdidase (units min^{-1}) within the digestive gland, gill and all remaining tissues (remainder) of slow- (< 2 g dry soft tissue) and fast-growers (> 2 g dry soft tissue) from the GENEPHYS G1 generation that had been reared in the natural environment at Bouin (Stock R.1). Data are the means ± 2 SE (n = 20). Enzyme activities are weight-corrected for a standard oyster of 25 mg soft tissue protein. Each oyster was bred from parents from Bonne Anse alone.

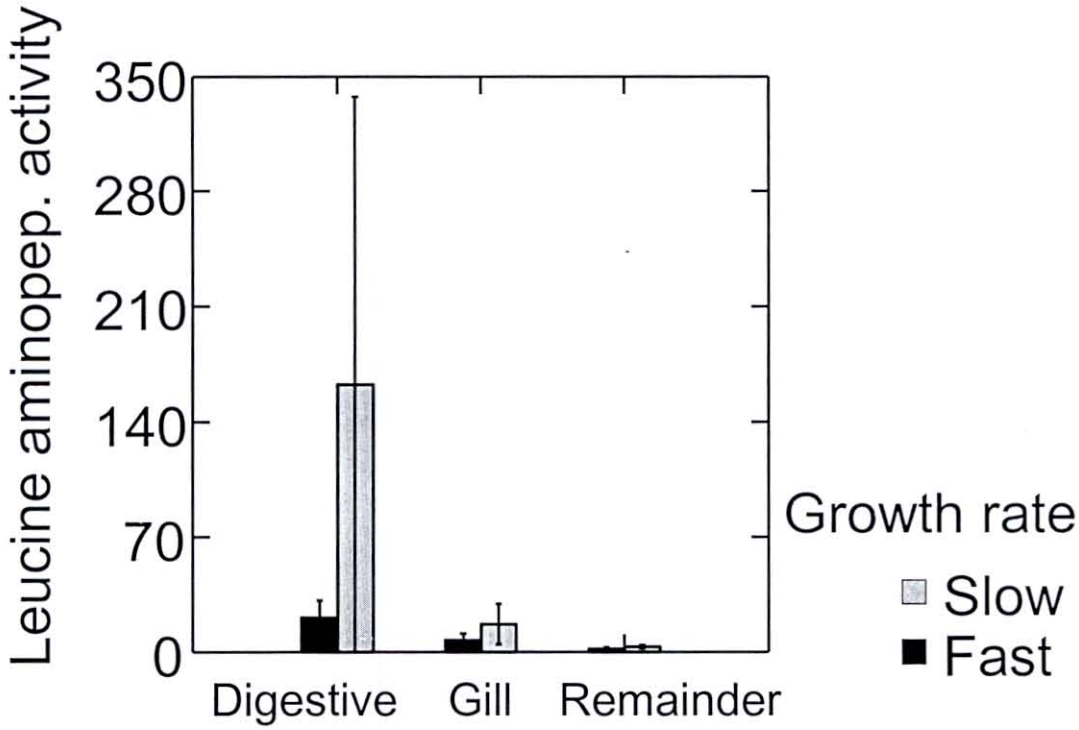
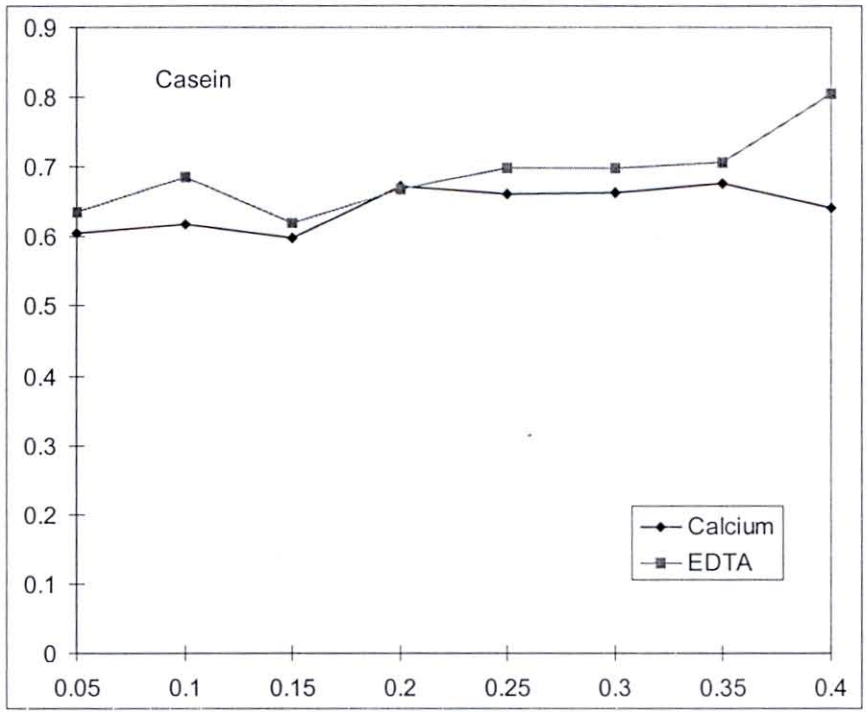


Figure 11. Crassostrea gigas. Net activities of calcium-dependent proteases in the eluate fractions from eight elution buffers that varied between 0.05 M and 0.4 M salt using either casein or oyster muscle protein as a substrate.



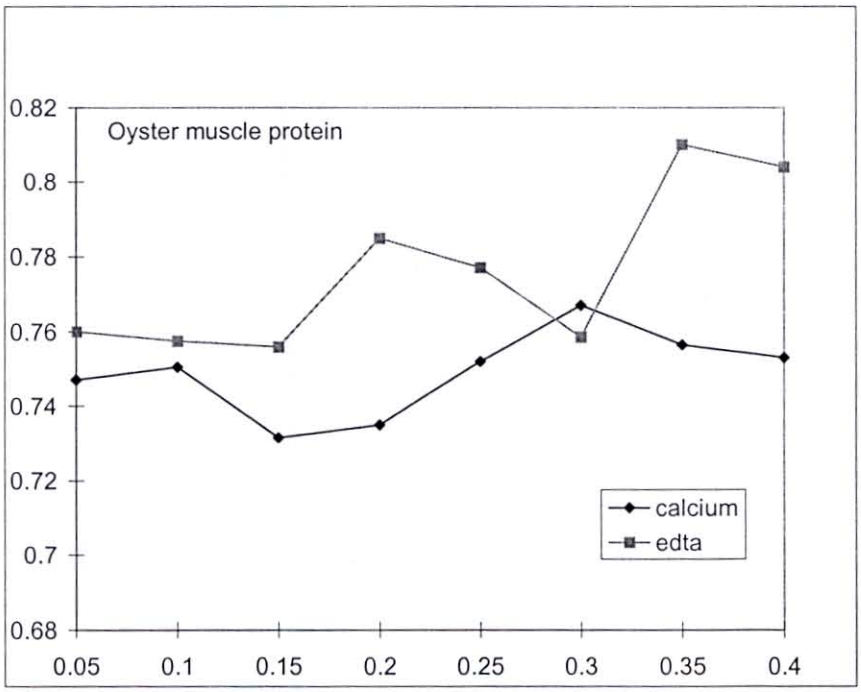


Figure 12. Crassostrea gigas. Ph profile for the net activity of calcium-dependent proteases, depicted as relative optical density (OD).

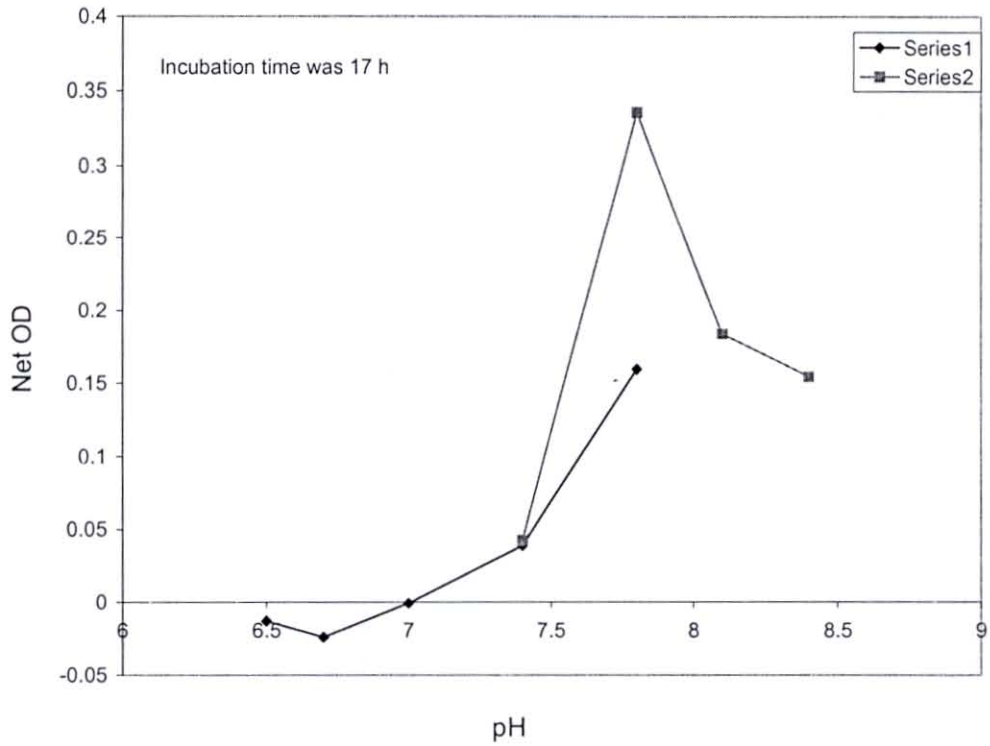


Figure 13. The timecourse of net activity for calcium-dependent proteases, measured at pH 7.8 on muscle protein, over two intervals that spanned from (a) 0 to 18 h and (b) from 0 to 1.75 h.

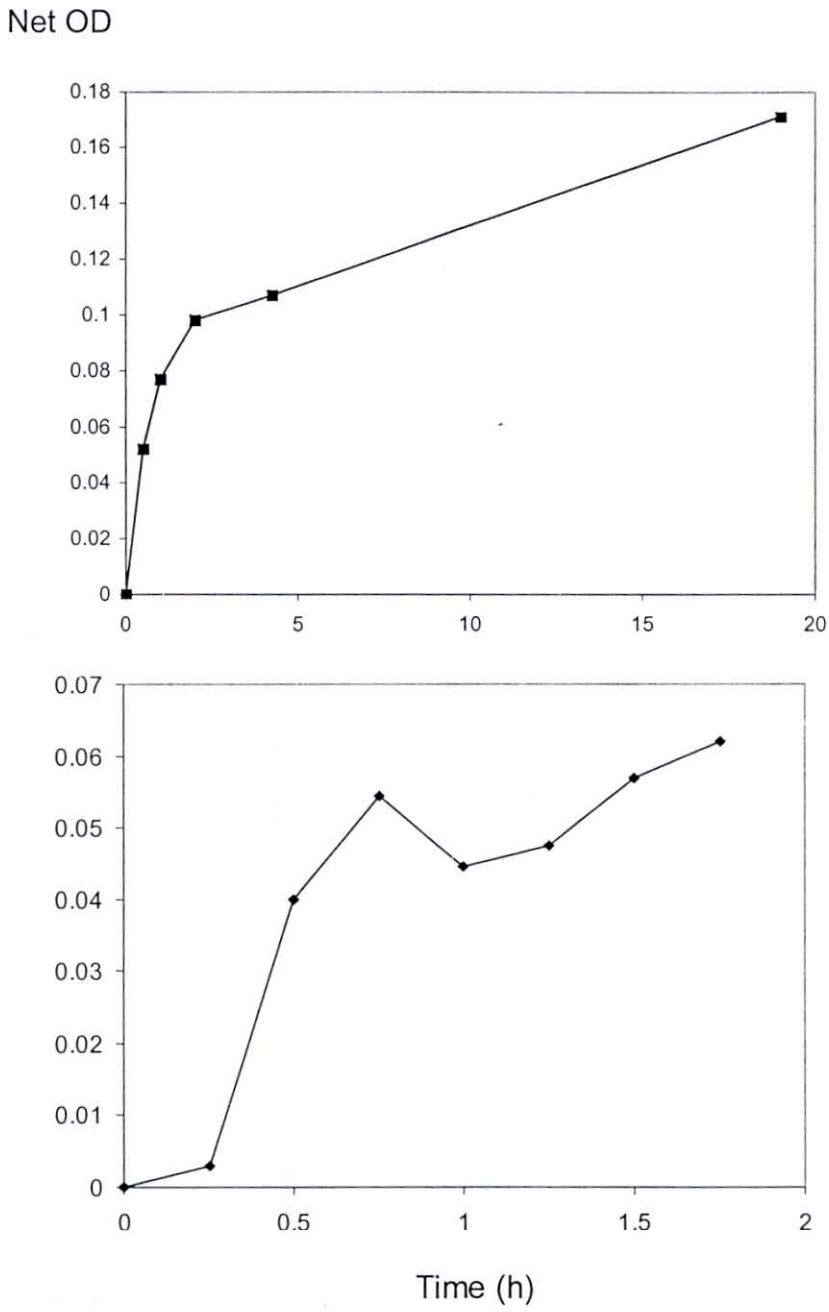
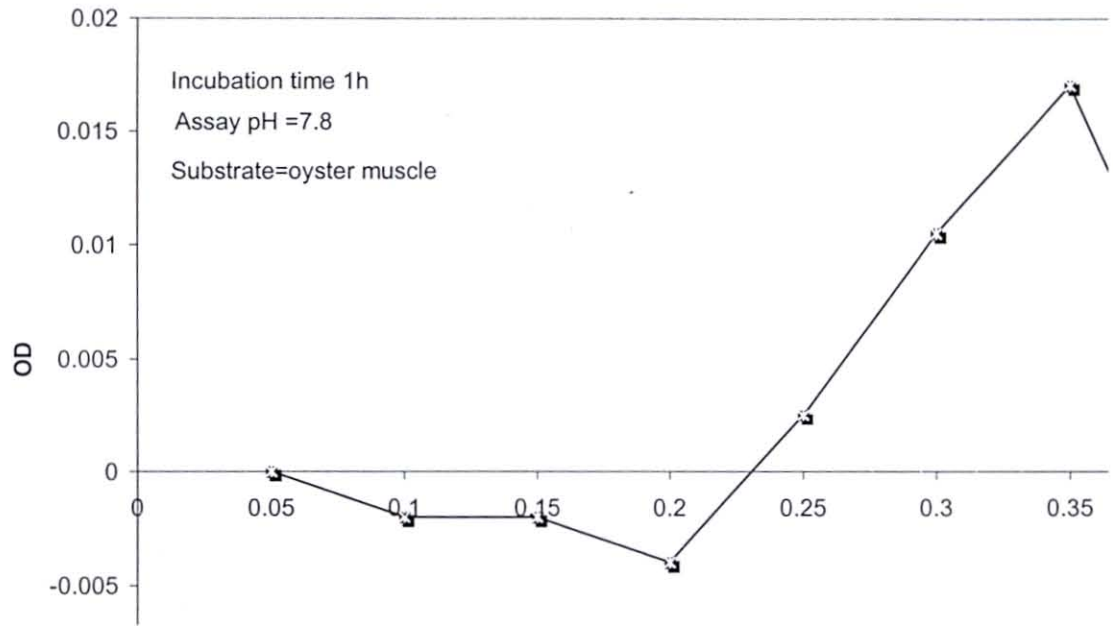


Figure 14. Net activity of calcium-dependent proteases in purified eluate fractions that contained from 0.05 M salt to 0.4 M salt.



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 1998

Partner n°3

CNRS

Centre National de la Recherche Scientifique

Observatoire Océanologique de Villefranche sur Mer

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual Progress Report
Participant n°3

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 1998

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**

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°3

CNRS UMS 0829 Contractor Dr. Catherine THIRIOT

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

ANNEX III
Individual Progress Report
Participant n°3

FAIR PL. 95.421

Individual Progress Report

for the period from
1st January to 31st December 1998

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	Obtaining 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	Karyotype 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 aneuploids/ PR			FR
5.4	Embryos analysesG1 / PR	Adults analysesG1 / PR	Adults analysesG1 Study of the aneuploidy transmission/ PR	Study of the aneuploidy transmission/ P R	Study of the aneuploidy transmission/ FR
8		Data processing/ PR	Data processing/ PR	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
FR = Final Report

Research activities during the third reporting period

Task 2 : Obtainment and management of the G2 generation

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

Evaluation of aneuploidy of the parents

Because a non-destructive method (i.e.biopsy) was not successful (see report 1997), a destructive method was used.

The following parents were selected from Genephys broodstocks and from Scottish broodstocks which have shown different levels of aneuploidy.

ANNEX III
Individual Progress Report
Participant n°3

1: Scotland female
2. Scotland female
3. Scotland female
4. Genephys female
5. Genephys female
6. Genephys female
7. Scotland male
8. Scotland male
9. Scotland male
10. Genephys male
11. Genephys male
12. Genephys male

The following crosses were performed:

Group 1:	Group 2:	Group 3:
female 4 x male 7	female 2 x male 11	female 6 x male 12
female 1 x male 7	female 5 x male 11	female 3 x male 12
female 4 x male 10	female 2 x male 8	female 6 x male 9
female 1 x male 10	female 5 x male 8	female 3 x male 9

After crossing (La Tremblade, July 1998), animals were processed for aneuploidy study by a destructive method (incubation in colchicine, dissection of gills, hypotonic treatment and fixation). Because one cross of group 1 was not successful, chromosome preparations were made for the 8 remaining animals.

Scoring of aneuploidy in these animals gave the following results:

Studied parents	no.slides observed	total mitosis	no.cells 2n=20	no.aneuploid cells 2n=19	2n=18	2n=17	total	% aneuploidy
2: scotland female	10	6	4	1	0	1	2	
3: scotland female	7	30	27	0	2	1	3	**10%**
5: genephys female	10	30	23	2	3	2	7	**23%**
6: genephys female	10	7	5	2	0	0	2	
8: scotland male	10	30	28	1	1	0	2	**7%**
9: scotland male	22	30	24	3	3	0	6	**20%**
11: genephys male	28	30	25	4	1	0	5	**20%**
12: genephys male	18	30	28	2	0	0	2	**7%**

It should be noted that:

(i) mitosis are very difficult to get in adult animals. We only observed six animals with 30 countable mitosis, which is our usual statistical number. In growing animals, this number is obtained with only one or two slides.

(ii) assessment of individual aneuploidy is not always representative of the aneuploidy of the population.

However, next spring it will be possible to study the progeny of four crosses: pure Genephys (female 5 x male 11), pure Scotland (female 3 x male 9) and mixed Genephys -Scotland (female 3 x male 12 and female 5 x male 8).

Task 5 : Genetical analyses

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

1. Study of aneuploidy in a control population originating from Argenton (Scotland broodstocks).

Animals of two size classes were scored for aneuploidy.

Small animals:

Animal #	Total of studied mitosis	no. cells 2n=20	no. aneuploid cells 2n=19	2n=18	2n=17	Total aneuploid cells
1	30	22	1	1	0	8
2	30	27	2	0	1	3
3	30	21	4	3	2	9
4	30	27	2	1	0	3
5	30	27	2	1	0	3
6	30	28	1	0	1	2
7	30	27	3	0	0	3
8	30	26	4	0	0	4
9	30	24	2	2	2	6
10	30	26	3	1	0	4
11	30	27	1	2	0	3
12	30	25	4	0	1	5
13	30	21	1	6	2	9
14	30	28	1	0	1	2
15	30	21	6	1	2	9

Aneuploidy was 16% in small animals.

Large animals:

Animal #	Total of studied mitosis	no. cells 2n=20	no. aneuploid cells 2n=19	2n=18	2n=17	Total aneuploid cells
1	30	29	1	0	0	1
2	30	30	0	0	0	0
3	30	30	0	0	0	0
4	30	28	1	1	0	2
5	30	30	0	0	0	0
6	30	28	0	1	1	2
7	30	29	1	0	0	1
8	30	27	1	1	1	3
9	30	27	2	1	0	3
10	30	28	2	0	0	2
11	30	29	1	0	0	1
12	30	26	2	0	2	4
13	30	28	1	1	0	2
14	30	29	1	0	0	1
15	30	28	1	0	1	2

Aneuploidy was 5% in large animals.

Among all the animals studied in this population, the total percentage of aneuploidy was 11%.

These results confirm the negative correlation between somatic aneuploidy and growth and the low level of aneuploidy of this population.

2. Study of aneuploidy in the Bouin population, studied simultaneously for growth and enzymes.

Tagged animals of three size class were studied, 12 small, 12 medium and 12 large.

ANNEX III
Individual Progress Report
Participant n°3

animal #	Total of studied mitosis	no. cells	no. of aneuploid cells			Total no. aneuploid cells
		2n=20	2n=19	2n=18	2n=17	
Small						
2120	30	24	2	3	1	6
2119	30	24	6	0	0	6
2013	30	22	7	1	0	8
2009	30	21	5	4	0	9
2315	30	23	6	0	1	7
2213	30	25	1	2	2	5
2212	30	21	6	2	1	9
2218	30	26	3	1	0	4
2211	30	25	0	3	2	5
2012	30	24	2	3	1	6
2217	30	22	2	4	2	8
2113	30	24	4	2	0	6
Medium						
2419	30	25	4	1	0	5
2511	30	20	7	2	1	10
2618	30	23	4	3	0	7
2514	30	26	2	2	0	4
2519	30	24	4	1	1	6
2014	30	26	3	1	0	4
2518	30	22	3	4	1	8
2719	30	24	3	3	0	6
2311	30	28	2	0	0	2
2612	30	26	4	0	0	4
2611	30	26	2	0	2	4
2818	30	30	0	0	0	0
Large						
2812	30	28	2	0	0	2
2711	30	28	1	1	0	2
2614	30	28	1	1	0	2
2516	30	28	1	0	1	2
2910	30	28	1	1	0	2
2716	30	29	1	0	0	1
2811	30	28	2	0	0	2
2814	30	28	2	0	0	2
2908	30	28	2	0	0	2
2714	30	27	2	0	1	3
2813	30	30	0	0	0	0
2909	30	27	2	1	0	3

Aneuploidy was 22% in small animals, 17% in medium animals and 6% in large animals.

The negative correlation between aneuploidy level and growth rate, which has been individually measured by partner 1, is particularly striking.

3. Study of aneuploidy in a group of full-sib families to assess the relation between aneuploidy and growth within and between families.

21 full-sib families were produced by Partner 1 to study the genetic basis of adaptation to environmental variability over time.

For 100 animals, individual weight was measured in June 1998. The mean within-family, and the mean between-families weights (i.e. mean for all families scored) were obtained.

Six families were selected according to different mean weight values.

Family	Mean weight (gr.) within-family	Mean weight (gr.) between-family
10	0.590	0.983
18	0.751	0.983
19	0.906	0.983
3	1.012	0.983
22	1.222	0.983
24	1.488	0.983

In each family, 30 animals with the mean within-family weight and 30 animals with the mean between-families weight (total: 330 animals) were treated for chromosome study (July and August 1998). As a first step, 10 animals per set will be studied.

Up to now, 35 animals have been scored for aneuploidy.

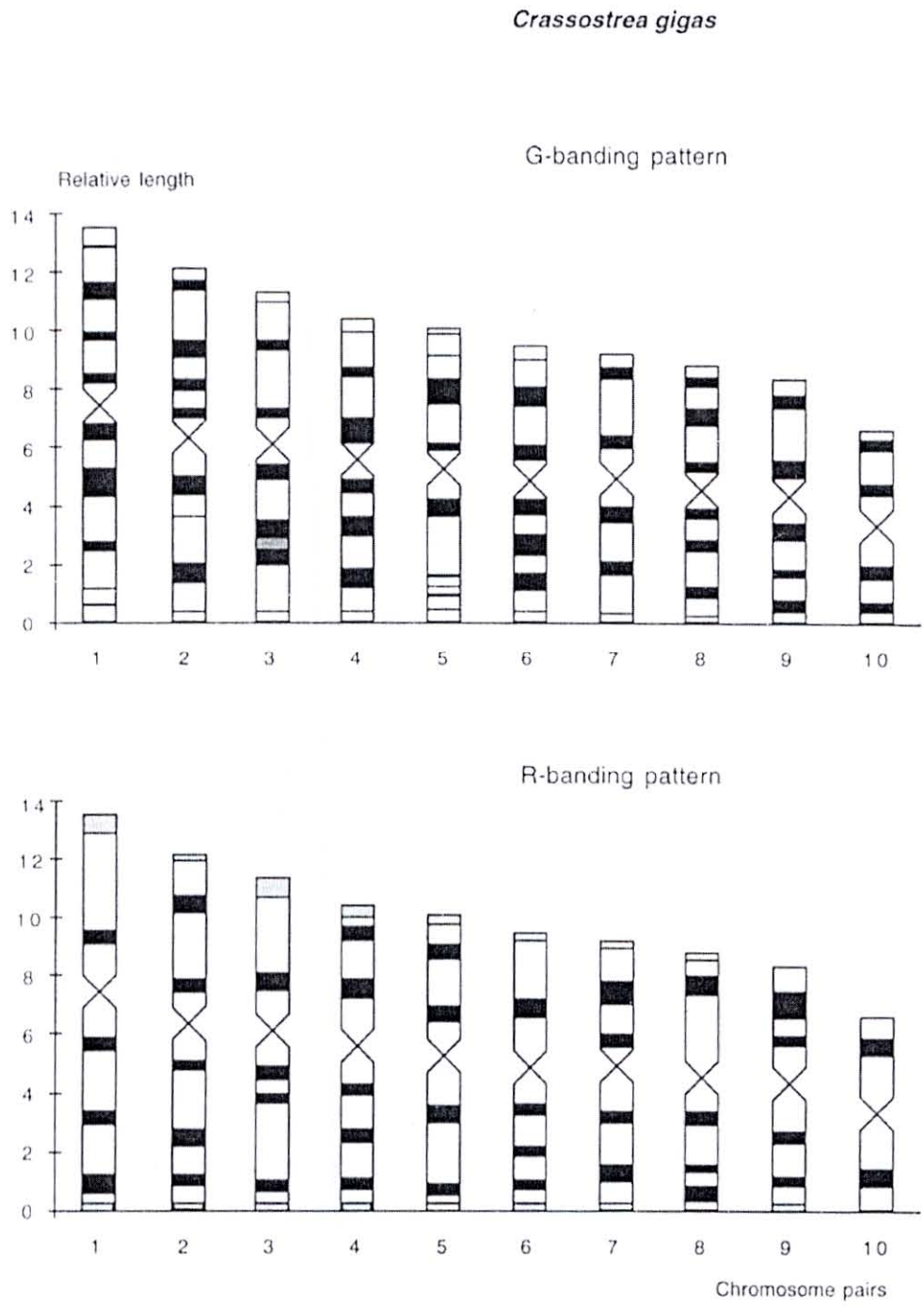
4. Identification of chromosomes by G and R banding.

In order to complete the preliminary results obtained last year on G and R banding patterns in *Crassostrea gigas*, more experiments were performed.

29 additional G-banded karyotypes were studied. This allowed us to distinguish more bands and to identify all chromosome pairs with the G-banding techniques.

Only 7 R-banded karyotypes could be obtained. Positive R-bands were always located in negative G-bands, which confirms that these two techniques are complimentary (see Figure in annex).

ANNEX III
Individual Progress Report
Participant n°3



Annex Figure of banding patterns in Crassostrea gigas

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 1998

Partner n°4

UCG

University College Galway

Departement of Zoology

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual Progress Report
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 1998**

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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Contractor Dr. Noël WILKINS

ANNEX III
Individual Progress Report
Participant n°4

FAIR PL. 95.421

Individual Progress Report

for the period from

1st January to 31st December 1998

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 / PR		FR
7	Perfectionment of the technique / PR	Perfectionment and application / PR	Aneuploïds characterisation / PR	Aneuploïds characterisation / PR	Aneuploïds characterisation / FR
8		Data processing/ PR	Data processing/ PR	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
FR = Final Report

Research activities during the third reporting period

Task 5 : Genetical analyses
Sub-task 5.1 : Allozymes

1. Introduction

The aim of sub-task 5.1 is to study the allozymes of the Go and G1 oysters *Crassostrea gigas* from the GENEPHYS program. The parents Go and the juveniles G1 were studied in the previous report. This report presents the results for the G1 when they had become adults. Departures from Mendelian expectations, the correlation between heterozygosity and growth and heterozygote deficiency were investigated.

The results obtained with the allozymes for the G1 juveniles were compared with the microsatellite results obtained by partner 5 (Institute of Marine Biology of Crete, Iraklion, Greece) . The results of this comparison are published here.

2. Materials and Methods

Thirteen allozymic loci were examined : Pgm, Lap, Idh-2, Est-1, EstD-2, EstD-3, Tap, Dap, Dia, Aat, Acon-1, Acon-2 (Table 1). The G1 adults were sampled in December 1997. They were then 21 months old. The sample came from both nurseries : La Tremblade and Bouin.

2.1 Parental contribution

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 offspring 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,1996). Departures of offspring genotype proportions from the expected values were tested using the χ^2 goodness-of-fit test. A comparison was done between the oysters as juveniles and as adult samples using a χ^2 goodness-of-fit test.

2.2 Correlation of heterozygosity and growth

The number of allozymes studied increased in comparison with the numbers studied in the juveniles. Six new allozymes loci were used : Tap, Dap, Dia, Aat, Aco-1, Aco-2 (Table 1).

The expected genotypes in the adult sample were calculated as for the juvenile sample (2nd progress report, 1997) according to the parental genotypes.

The correlation between heterozygosity and growth was calculated using different parameters. For heterozygosity, two parameters, Hie and Ho, were used. Hie is the heterozygosity class i.e. the number of heterozygous loci for each individual. Ho means observed heterozygosity i.e. Hie divided by the total number of loci really studied. Ho allowed us to use individuals which were not genotyped for all loci.

The growth parameters were defined by

- the Initial weight (IW) : the total weight of each oyster at the beginning of the experiment.
- the Final weight (FW) : the total weight of each oyster at the end of the experiment.
- the Growth slope (GS) : regression slope of the weight vs time. The growth was linear during the experiment so the growth slope represents the growth rate for each individual.

To summarise

→Heterozygosity parameters :
- **Hie** : heterozygosity classes
- **Ho** :observed Heterozygosity

→Growth parameters
- **IW** : Initial Weight
- **FW** : Final Weight
- **GS** : Growth Slope

Enzyme Name		Ec Enzyme No	Electrophoretic buffer	Tissue	Loci screnned
Phosphogluco mutase	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
Tri-Amino-Peptidase	TAP		Tris Citrate (TC) 8.0	Muscle	TAP
Di-Amino-Peptidase	DAP		Tris Citrate (TC) 8.0	Muscle	DAP
Diaphorase	DIA	1.6.4.3	Tris Citrate (TC) 8.0	Digestive Gland	Dia
Aspartate-Amino-Transferases	AAT	2.6.1.1	Tris Citrate (TC) 8.0	Digestive Gland	Aat
Aconitase	ACO	4.2.1.3	Tris Citrate (TC) 8.0	Digestive Gland	Aco-1, Aco-2

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

The correlation between heterozygosity and growth were done using these five parameters. Linear regressions were used to know if the correlations were significant.

An ANOVA was performed to compare homozygotes and heterozygotes at each locus for the 3 populations.

The differences between the weights of the different genotypes at each locus were also calculated by an ANOVA.

2.3 Heterozygote deficiency

The Hardy-Weinberg equilibrium was tested as previously described for the juvenile sample with the software GENEPOP (Raymond and Rousset, 1995). Moreover the heterozygote deficiency was calculated for each growth class using the growth slope parameters with the Selander index D. The total frequency of homozygotes is expected to be equal to Σipi^2 . The frequenciy of heterozygotes will then equal :

$$He= 1-\Sigma ipi^2$$

An usual measure of the departure from HW equilibrium is :

$$D = \frac{Ho-He}{He}$$

Where Ho is the observed heterozygosity. Positives values of D indicate an excess of heterozygotes and a negative value indicate an deficit of heterozygotes (Singh, 1980).

3 Results

3.1 Parental Contribution.

3.1.1 Allozymic Frequencies

Allelic frequencies of the Go parents , the G1 juveniles and the G1 adults for the 3 populations are presented in Table 2. No new alleles (ie alleles not observed in the G0 parents) were found either in the juveniles or in the adults, so it seems there are no foreign individuals contaminating the experimental families.
Six new polymorphic loci were studied for the adult sample. They had a mean of 3.83 alleles per locus in the 3 populations (Table 3). .

- Port des Barques populations.

The allozymic frequencies found in the juveniles and in the adults did not change a lot. In Idh-2, a 85 allele was not present in the juveniles but it was found at low frequency in the adult samples.

- Bonne Anse

A difference between allozymic frequencies was found at the Est-1 locus. In the juveniles, the 98 allele was the major allele (fequency=0.58) but in the adults sample it was the 100 allele which was the major one. From the parental values, both alleles, 98 and 100, were expected to show the same frequency.

- Arcachon

For the Pgm locus, the allelic frequency of the adult samples was closer to the parents than the juveniles samples. It is the same pattern for the EstD-2 locus. For the EstD-3 locus, the 95 allele was found in the adults with a slightly higher frequency than in the parents whereas it was not found in the juveniles samples.

		Port des Barques			Bonne Anse			Arcachon		
		Parents	Juveniles	Adults	Parents	Juveniles	Adults	Parents	Juveniles	Adults
Pgm	N	10	50	81	10	49	49	10	45	67
	85	0.05	0.02	0.043						
	90	0.05	0.13	0.123						
	95				0.2	0.173	0.148	0.1	0.044	0.067
	100	0.4	0.36	0.487	0.4	0.489	0.591	0.65	0.844	0.716
	102	0.15	0.12	0.117	0.25	0.184	0.132	0.1	0.033	0.154
	110	0.3	0.24	0.167	0.05	0.102	0.112	0.15	0.078	0.06
	115	0.05	0.13	0.062	0.1	0.05	0.02			
Lap	N	10	50	81	10	50	49	10	48	66
	95	0.111	0.03	0.049	0.25	0.08	0.081	0.15	0.083	0.025
	100	0.5	0.59	0.685	0.6	0.87	0.867	0.55	0.718	0.856
	105	0.222	0.23	0.129	0.15	0.05	0.05	0.3	0.194	0.114
	110	0.167	0.15	0.135						
Gpi	N	10	50	81	10	50	49	10	48	67
	90				0.1	0.04	0.031	0.15	0.031	0.075
	100	1	1	1	0.55	0.88	0.898	0.75	0.927	0.835
	110				0.35	0.08	0.071	0.1	0.042	0.089
Idh-2	N	10	49	77	10	49	50	10	48	67
	85	0.05	0	0.007						
	90	0.1	0.061	0.07				0.2	0.187	0.082
	95	0.2	0.061	0.05	0.3	0.234	0.29	0.05	0.031	0.048
	100	0.5	0.602	0.675	0.6	0.745	0.7	0.6	0.697	0.731
	110	0.15	0.275	0.195	0.1	0.02	0.01	0.15	0.083	0.142
Est-1	N	10	49	81	10	50	50	10	50	67
	98	0.2	0.114	0.08	0.4	0.58	0.35	0.35	0.231	0.246
	100	0.45	0.555	0.605	0.4	0.34	0.55	0.5	0.653	0.672
	102	0.3	0.291	0.29	0.2	0.08	0.1	0.15	0.105	0.082
	104	0.05	0.04	0.025						
EstD-2	N	10	45	70	10	49	44	10	48	56
	100	0.5	0.488	0.41	0.3	0.265	0.136	0.25	0.167	0.276
	105	0.5	0.512	0.59	0.6	0.694	0.761	0.65	0.771	0.607
	110				0.1	0.041	0.102	0.1	0.062	0.116
EstD-3	N	10	47	81	10	50	47	10	48	67
	95	0.1	0.021	0.031				0.05	0	0.067
	100	0.9	0.979	0.969	1	1	1	0.8	0.948	0.813
	105							0.15	0.052	0.119

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

		Port des Barques	Bonne Anse	Arcachon
		Adults	Adults	Adults
Tap	N	80	47	63
	96	0.075	0.02	
	98	0.125	0.122	0.059
	100	0.688	0.786	0.816
	102	0.094	0.051	0.11
	104	0.019	0.02	0.015
Dap	N	71	47	66
	96	0.342	0.128	0.136
	98	0.368	0.394	0.265
	100	0.243	0.351	0.485
	102	0.046	0.128	0.114
Dia	N	77	47	68
	94	0.084	0.181	0.14
	96	0.26	0.362	0.154
	100	0.539	0.415	0.64
	102	0.11	0.032	0.059
	104	0.006	0.011	0.007
Aat	N	81	50	69
	90	0.259	0.03	0.043
	100	0.469	0.576	0.406
	110	0.272	0.394	0.551
Aco-1	N	80	48	67
	98	0.1	0.281	0.485
	100	0.813	0.719	0.425
	102	0.087		0.09
Aco-2	N	80	84	67
	96			0.015
	98	0.094	0.156	0.097
	100	0.863	0.792	0.687
	102	0.044	0.052	0.201

Table 3 : Allelic frequency for the loci studied on the adult samples.

3.1.2 Allozymic genotypes

The difference in genotype proportions between the Go parents, the G1 juveniles and the G1 adults was tested by a χ^2 goodness-of-fit test. The results are summarised in Table 4.

- Port des Barques

  Strong significant differences in the genotype proportions between parents and juveniles were found and between the parents and the adults for all loci. The probability of significant difference decreased to 0.05 at EstD-2 locus.

  One significant difference was found between the juveniles and the adults in the EstD-2 locus.

- Bonne Anse

  Strong significant differences in the genotype proportions between parents and juveniles were found and between the parents and the adults for all loci. The probability changed in 3 cases. The probability decreased to 0.05 at the Idh-2 locus and at the Est-1 locus between the parents and the adults.

No difference was found between the juveniles and the adults.

♦ Arcachon

When a difference was found between the parents and the juveniles, it was found also between the parents and the adults except for the Idh-2 and the EstD-3 loci. For the Gpi locus a difference was found in the both case but not with the same probability of significance. The difference between the parents and the juveniles was more significant that than between the parents and the adults. For the Idh-2 locus, no significant difference was found between the parents and the juveniles but one was present between the parents and the adults. For the EstD-3, it was the contrary, a significant difference was only found between the adults and the juveniles.Between the juveniles and the adults, two significant differences were found for the Pgm locus at a 0.05 probability and for the EstD-3 locus at 0.001 probability.

	Arcachon			Bonne Anse			Port des Barques		
	par/juv	par/adu	juv/adu	par/juv	par/adu	juv/adu	par/juv	par/adu	juv/adu
Pgm	S***	S***	S*	S***	S***	NS	S***	S***	NS
Lap	S***	S***	NS	S***	S***	NS	S*	S*	NS
Gpi	S***	S*	NS	S***	S***	NS			
Idh-2	NS	S*	NS	S***	S*	NS	S***	S***	NS
Est-1	S***	S***	NS	S***	S*	NS	S***	S***	NS
EsD-2	NS	NS	NS	S*	S***	NS	S***	S*	S*
EsD-3	S**	NS	S***				S*	S*	NS

Table 4: Difference in genotypic proportions between juveniles and their parents in the 3 populations. The expected numbers of genotypes were calculated as described in materials and methods. (S=p<0.05 ; S** = p<0.01 ; S*** =p<0.001)*

Microsatellite genotypes.

The microsatellite markers allowed us to identify the parents of almost all offspring. The contribution was very heterogeneous between parents.

♦ Port des Barques population

The parents of only one juvenile could not be identified by microsatellites and allozymes markers. The identification of parents by microsatellites was in agreement with allozymes for almost all juveniles. Some difference were found for individuals which genotyped as homozygotes for the Pgm and Lap loci even though they should be heterozygotes according to the parental genotypes. For these individuals, the single allele that they carry was always one present in a parent.

♦ Bonne Anse population

The kinship of only one juvenile could not be proven because that individual was genotyped for only one microsatellite markers. The microsatellite genotypes were in agreement with almost all allozymic genotypes in all other individuals.

♦ Arcachon population

All juveniles' parents were identified by the 3 microsatellite markers. The allozyme genotypes were in agreement with microsatellites genotypes except for a small numbers of genotypes which were read as homozygotes even though they must be heterozygotes in accordance with the parental genotypes.

3.2 Relationship between Heterozygosity and Growth

3.2.1 Relationship between heterozygosity and growth parameters

The relationship between heterozygosity and growth was studied in the 3 populations (Arachon, Bonne Anse, Port des Barques) for two heterozygous traits (Hie and Ho) and 3 growth parameters (IW, FW, GS). For each population, two sub-samples were examined. Sub-samples corresponded to the groups called Bouin and La Tremblade. Table 5 gives the coefficient of detemination (R^2) and the probability of significance.

*PORT DES BARQUES

		R^2 values		
		Initial weight	growth slope	final weight
Total population	Hie	.014 NS	.0207NS	.0166NS
	Ho	.01NS	.019NS	.017NS
Population6 La Tremblade	Hie	.055NS	.0402NS	.0450NS
	Ho	.04NS	.050NS	.056NS
Population 2 Bouin	Hie	.0217 NS	.04NS	.040NS
	Ho	.016NS	.020NS	.0131NS

*BONNE ANSE

		R^2 values		
		Initial weight	growth slope	final weight
Total population	Hie	**.111S**	.000NS	.007NS
	Ho	**.203S**	.000NS	.009NS
Population 7 La Tremblade	Hie	**.165S**	.0139NS	.002NS
	Ho	**.196S**	.0048NS	.000NS
Population3 Bouin	Hie	.095NS	.003NS	.008NS
	Ho	**.200S**	.010NS	.030NS

*ARCACHON

		R^2 values		
		Initial weight	growth slope	final weight
Total population	Hie	.023NS	.007NS	.006NS
	Ho	.047NS	**.060S**	**.066S**
Population8 La Tremblade	Hie	.005NS	.0011NS	.017NS
	Ho	.001NS	.000NS	.0065NS
Population 4 Bouin	Hie	.000NS	.000NS	**.290S**
	Ho	.081NS	**.140S**	**.150S**

Table 5: Coefficient of determination (R^2) and its significance (S: significant, NS: non significant) in correlations between various growth parameters (IW, GS, FW)and two measures of heterozygosity (Hie = heterozygosity classes, Ho = observed heterozygosity). Statistically significant values are shown in bold.

Regression of individual growth and multilocus heterozygosity revealed no significant correlation in the Port des Barques population.
In the Bonne Anse population, the initial weight (IW) exhibited a significant, negative correlation with Ho and Hie in the whole population and in the subsamples (7 and 3

grown in La Tremblade and in Bouin respectively). The only exception was the Hie/IW in the Bouin group (3) which was negative but not significant (Figure 1).

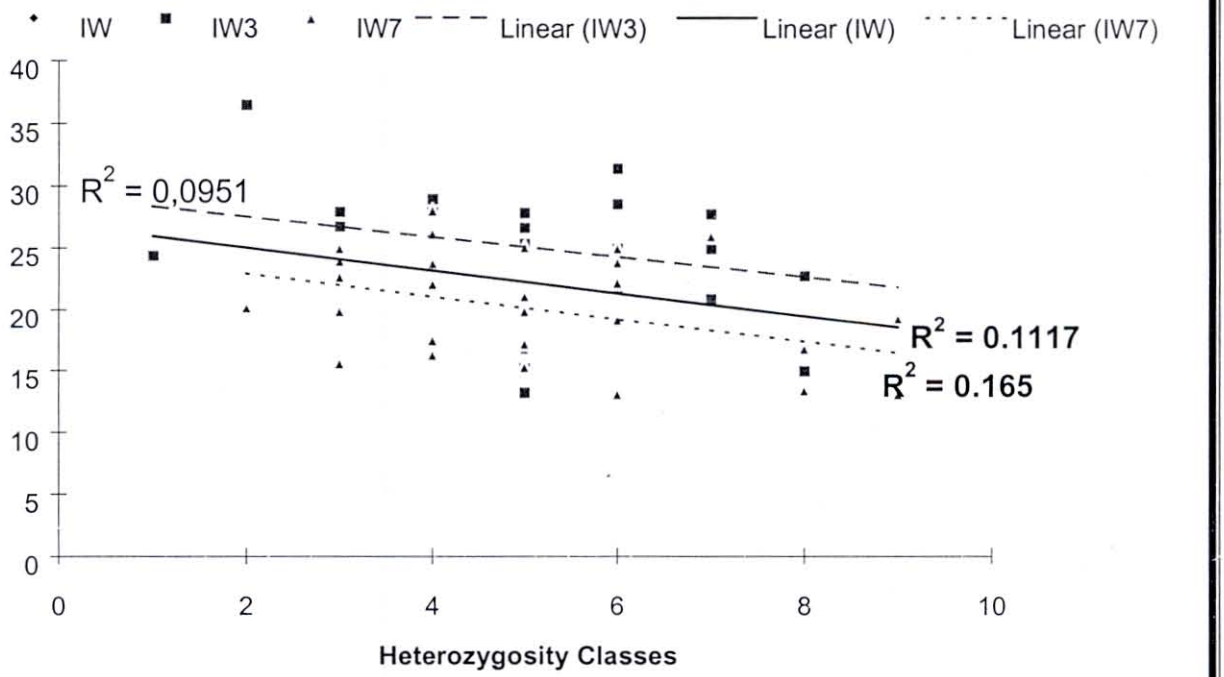

Figure 1: Correlation between heterozygosity classes and initial weight in the Bonne Anse population.

In the Arcachon population, negative correlations were found between heterozygosity classes(Hie)/growth slope (GS) and observed heterozygosity(Ho)/final weight (FW) but not between heterozygosity classes(Hie)/growth weight (GW) or final weight (FW). The probability for the regression between Initial weight (IW) and observed heterozygosity (Ho) was close to significance (p=0.063 for the total population and p=0.06 for the subsample 4).When the Arcachon population is split in two subsamples , pop 4 (Bouin) and pop 8 (La Tremblade), (See Materials and Methods), there was a negative correlation between Ho/growth slope (figure 2) and Hie or Ho/Final weight (FW) in pop 4 (Bouin).

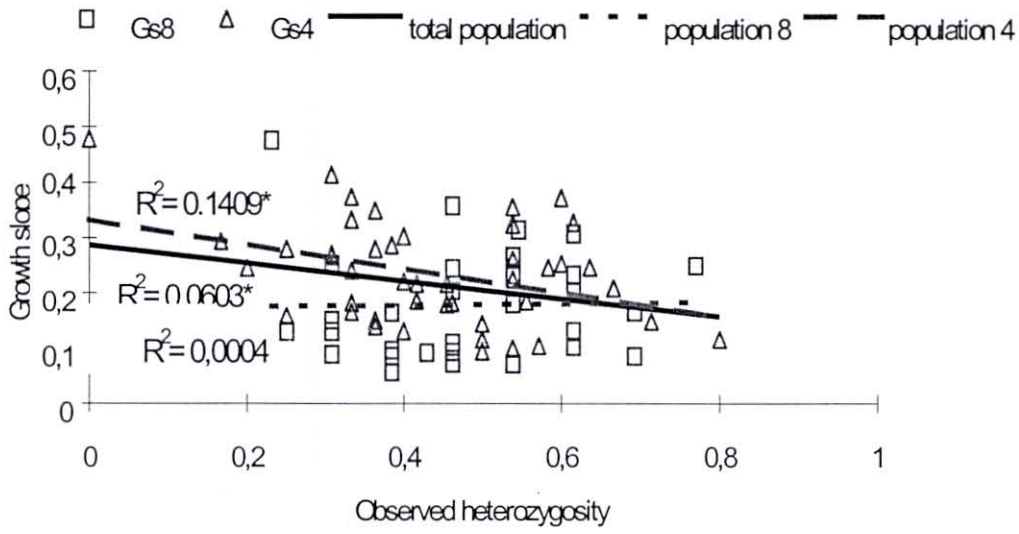

Figure 2: Regression observed heterozygosity (Ho) against Growth slope in Arcachon population.

Even if the correlation was not found in the 3 populations and for the 3 growth parameters, the negative correlation found for different parameters in different populations is unusual in bivalve studies.
To sum up : correlation between heterozygosity and growth are not clearly present in the 3 adults populations. The significant negative correlation found in this study were not replicable between growth parameters although they were linked by a strong correlation.
The Arcachon population showed a difference between the two sub-samples. There is a significant negative correlation between Ho /GS, between Ho / FW and between Hie/FW for the sub-sample 4 (Bouin) but not with the sub-sample 8 (La Tremblade) .

*Relation between heterozygosity classes and the growth parameters

When the means of each growth parameter (IW, GS, FW) are plotted for each heterozygosity class (figure 3), no correlations were found and the coefficients of variation did not decrease significantly when the number of heterozygous loci per individual increased. There was no correlation if the population was split into subsamples based on locality of ongrowing.

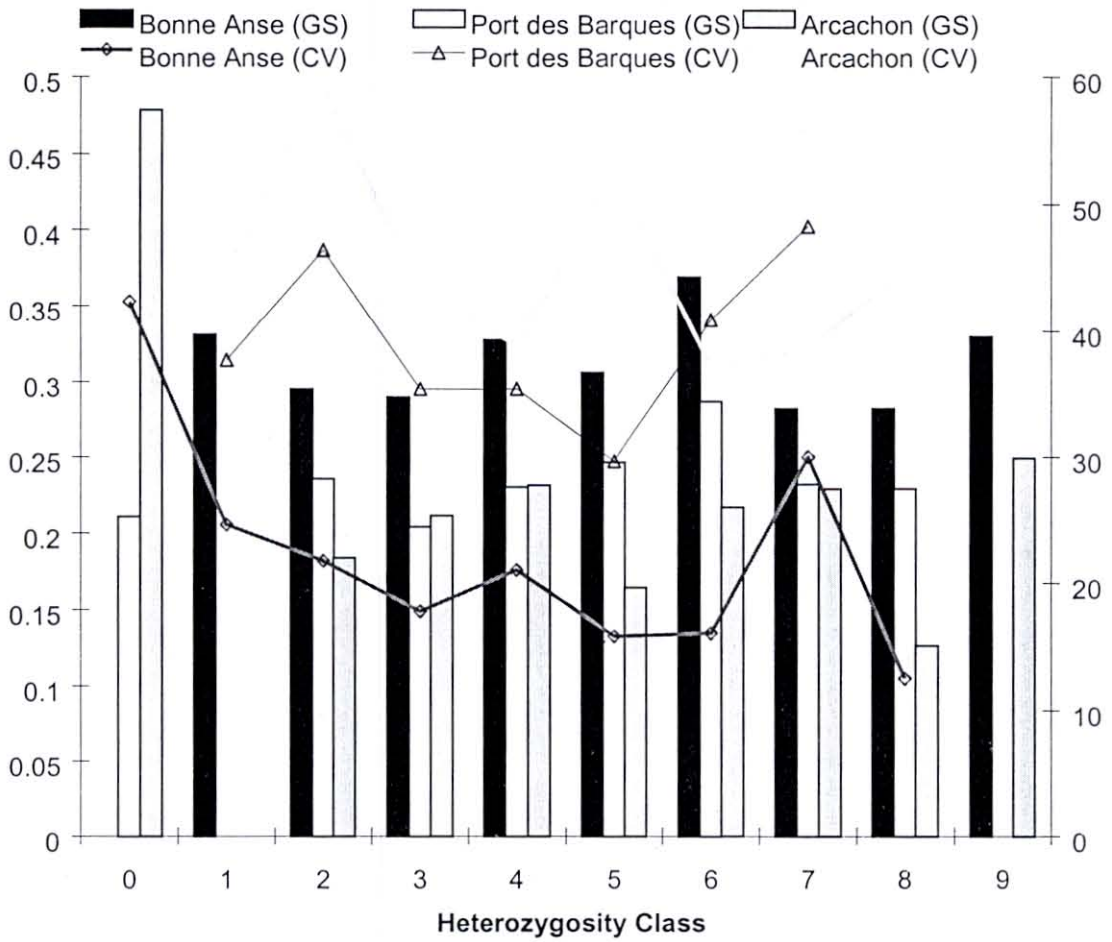

Figure 3: Relationship between each heterozygozity class and the mean of the Growth Slope (GS).

3.2.2 Differences between homozygotes and heterozygotes

The mean growth parameters (IW, GS, FW) of heterozygotes compared with those of homozygotes at each locus for all populations was tested using ANOVA .Table 6 summarises the result of the whole test. It indicates, in bold type, the instances where the groups are significantly different and which group, homozygotes or heterozygotes, is largest in each case.

	ARCACHON			PORT DES BARQUES			BONNE ANSE		
	IW	GS	FW	IW	GS	FW	IW	GS	FW
Pgm	NS	NS	NS	NS	NS	NS	NS	**S He>Ho**	**S He>Ho**
Lap	NS	NS	NS	NS	NS	NS	**S Ho>He**	NS	**S**
Gpi	NS	NS	NS				NS	**S Ho>He**	NS
Idh-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Estd-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Estd-3	**S Ho>He**	NS	**S Ho>He**	NS	**S He>Ho**	NS			
Tap	NS	NS	NS	NS	NS	NS	**S Ho>He**	NS	**S Ho>He**
Dap	NS	NS	NS	NS	NS	NS	NS	NS	NS
Dia	NS	NS	NS	NS	NS	NS	NS	**S Ho>He**	NS
Aat	NS	**S Ho>He**	NS	NS	NS	NS	NS	NS	**S He>Ho**
Aco-1	NS	NS	NS	NS	NS	NS	NS	NS	NS
Aco-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Est-1	NS	NS	NS	NS	NS	NS	**S Ho>He**	NS	NS

Table 6: Summary of the ANOVA results testing the mean growth parameters of heterozygotes (He) againt that of homozygotes (Ho). (IW: Initial weight; Fw: Final weight; GS: growth slope).

To sum up: no consistent difference was found in the 3 populations for the same locus. When significant difference were found, homozygotes were larger than heterozygotes in 9 out of 13 cases. When a difference was found for one of the growth parameters, it was never found for the two other parameters. For the Initial Weight, 4 significant differences were found , all showed homozygotes larger than heterozygotes. For the growth slope, five difference were found but only 3 out of 5 showed homozygotes larger than heterozygotes. And in the Final weight, only 2 differences out of 4 showed homozygotes higher than heterozygotes.

3.2.3 Relation between genotypes and the growth parameters

For each genotype, the mean growth parameters were tested using ANOVA (Table 7). When a significant difference was found between genotypes, an ANOVA was performed to compare the difference between each allele.

- Port des Barques populations.

Three loci showed a significant difference between genotypes : EstD-3, Aat, Est-1. The difference were found in Growth Slope for the EstD-3 locus and in Initial Weight for the Aat and the Est-1 loci. Significant difference were also found between allele at EstD-3 and at Est-1 for the growth parameters as well for the genotypes. The Aat locus did not show a significant difference between alleles and any growth parameters.

- Bonne Anse population.

Six loci showed significant differences between genotypes : Lap, Gpi, Idh-2, Tap, Dia, Aat loci. A significant difference was only found for one of the growth parameters, in IW for the Lap and Aat loci ; in Growth Slope for the Gpi, Idh-2 and Dia loci . A difference

was found in IW and FW for the Tap locus. The differences between alleles were then tested by an ANOVA. A significant difference between allele was found at the Gpi locus and at the Idh-2 locus for the Growth slope and at the Aat locus for Initial weight. For the Tap and Dia loci, no differences were found for the alleles even though significant differences were found for the genotypes. For the Lap locus, a significant difference was found in Initial weight as for genotypes but also in the Final Weight.

- Arcachon population.

Differences between genotypes were found at different loci : Pgm, EstD-3, Aat, Est-1. Significant differences were found in the growth parameters only at 3 loci (Pgm, EstD-3, Aat). Significant difference in Est-1 were only present in the Initial Weight (IW) and Final Weight (FW). When an ANOVA is performed on the weights associated with various alleles for the genotypes showing significant difference, a significant difference was found between the alleles. But at EstD-3, no significant difference was found for Growth Slope between alleles (the probability was close to significance, p=0.067).
Even for Est-1 where no significant difference was observed in Growth slope between genotypes there was a significant difference in Growth slope between alleles.

No loci showed a significant difference in genotypes or alleles for the all growth parameters in the three populations. Two loci showed a significant difference in two populations, EstD-3 and Est-1 and a significant difference in the Aat locus was present in the 3 populations for the Initial Weight.

◊ EstD-3 locus.

In the Arcachon population, both 100/100 and 95/100 genotypes had almost the same weight and were higher than 100/105 genotypes. In the Port des Barques population, the 95/100 genotype was higher than 100/100 but the 100/105 genotypes was not present in this population. Even though the 95 allele was associated with higher weight in both population than other alleles, the difference in the Arachon population did not seem to be significant.

◊ Est-1 locus

In the Arcachon population, the 100/100 genotypes are much lighter than the other genotypes. It was the opposite in the Port des Barques population where the 100/100 genotypes were higher than the other genotypes. It was the same pattern when the alleles were compared.

◊ Aat locus

When the genotypes were ranked according to their mean weight there was a difference in the ranking in the different populations.

	ARCACHON			PORT DES BARQUES			BONNE ANSE		
	IW	GS	FW	IW	GS	FW	IW	GS	FW
Pgm	**S**	**S**	**S**	NS	NS	NS	NS	NS	NS
Lap	NS	NS	NS	NS	NS	NS	**S**	NS	NS
Gpi	NS	NS	NS				NS	**S**	NS
Idh-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Estd-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Estd-3	**S**	**S**	**S**	NS	**S**	NS			
Tap	NS	NS	NS	NS	NS	NS	**S**	NS	**S**
Dap	NS	NS	NS	NS	NS	NS	NS	NS	NS
Dia	NS	NS	NS	NS	NS	NS	NS	**S**	NS
Aat	**S**	**S**	**S**	**S**	NS	NS	**S**	NS	NS
Aco-1	NS	NS	NS	NS	NS	NS	NS	NS	NS
Aco-2	NS	NS	NS	NS	NS	NS	NS	NS	NS
Est-1	**S**	NS	**S**	**S**	NS	NS	NS	NS	NS

Table 7: abstract of the ANOVA results testing the difference between genotypes for each locus. S in bold means Significant ; NS, Non Significant. The empty boxes are because the locus was monorphic in that population.

To sum up :

- Correlation between heterozygosity/growth :

⇒ A negative correlation between Observed Heterozygosity (Ho) and the Final Weight (FW) and between the Observed Heterozygosity (Ho) and the Growth Slope (GS) were found in the Arcachon population.

⇒ Differences in the correlation was found between the sub-samples (different nurseries) of the Arcachon population.

⇒ Negative correlations between Observed Heterozygosity (Ho) or Heterozygosity Classes (Hie) and the Initial Weight (IW) were found in the Bonne Anse population.

- Correlation between heterozygosity classes/ mean of the growth :

No significant correlation, neither between heterozygosity classes and the mean of the growth parameters nor between heterozygosity classes and the coefficient of variation was found.

- Difference between Homozygotes and Heterozygotes

No significant difference in growth parameters between homozygotes and heterozygotes was found for any one locus in all of the 3 populations.

- Relationship between genotypes or allele and the growth :

No significant relationship between genotypes or alleles with the growth was found in the 3 populations.

3.3-Heterozygote deficiency

Heterozygote deficiency was tested as described in Materials and Methods. The results of the probability that there is a departure from Hardy-Weinberg equilibrium are showed in table 8.

- Port Des Barques population.

The parents sample showed no deviations from HW expectations. On the other hand 4 loci out of 6 deviated from HW equilibrium in juveniles, in all cases it was a heterozygote deficiency. In the adult sample, Idh-2 and Est-1 loci showed a deficit as in the juvenile sample. Pgm and EstD-2 loci did not have a heterozygote deficiency in the adult sample. On the contrary, the Lap locus which did not show a heterozygote deficiency in the juvenile sample had a significant deficit in the adults sample. In the loci screened only in the adult sample there was only one locus showing a deficit : the Tap locus .

- Bonne Anse population.

Three out of 6 loci showed a deficit in the juvenile sample but only the Pgm locus kept this deficit in the adult sample. Many of the new loci screened for the adult sample showed a deficit viz. Tap, Dap, Dia and Aat.

- Arcachon population.

Almost no deficits were found in the parent and in the juvenile samples. A deficit for Est-1 was only present in the adult sample. The Dia locus which was only screened in the adult sample presented a deficit in heterozygotes also.

The deficit in heterozygotes in class of weight were analysed using the D index (see Materials and Methods). The deficit of heterozygotes for each Growth Slope (GS) class is represented in figure 4. The three populations did not show an increase or a decrease of departures from Hardy-Weinberg expectations between weight class.

To sum up : Heterozygote deficiency

- No consistent difference was found between juveniles and the adults for deficiency of heterozygotes in the 3 populations.
- The Lap locus showed a strong deficit in heterozygotes in the Port des Barques adults whereas no deficit was found in the juveniles.
- The new allozymes tested for the adults showed a strong deficit for 4 loci out of 6 in the Bonne Anse population.
- The heterozygote deficiency did not have a pattern of increase or decrease depending on the growth slope.

	Port des barques			Arcachon			Bonne Anse		
	Parent	Juvenile	Adult	Parent	Juvenile	Adult	Parent	Juvenile	Adult
Pgm	.4077	.0079**	.0619	1	.0855	.7408	.6568	.0000**	.0016**
Lap	.9052	.1589	.0002**	.1627	.2208	.7592	.5999	.5912	1
Pgi	-	-	-	1	1	1	.1131	.3324	1
Mdh-1	-	-	-	-	-	-	-	-	
Mdh-2	-	-	-	-	-	-	-	-	
Idh-1	-	-	-	-	-	-	-	-	
Idh-2	.5285	.0000**	.0096**	.871	.7657	.3927	.3598	.6724	.7849
Est-1	.6085	.0001**	.0030**	.3671	.0562	.0163*	.1687	.0159*	.7345
EstD-2	.1998	.0000**	.4681	.5046	.3874	.6861	.3598	.0040**	.1427
EstD-3	1	1	1	1	.1036	.5553	-	-	-
Tap			.0000**			.0928			.0314*
Dap			.4715			.4171			.0049*
Dia			.3619			.0151*			.0004** *
Aat			.1009			.2360			.0057**
Aco-1			.7757			.8683			1
Aco-2			.1686			.3119			1

Table 8: Probability (P value) that the genotypes were in Hardy Weinberg equilibrium in juveniles and in the parents for the 3 sites (p<0.05, ** p<0.01).*

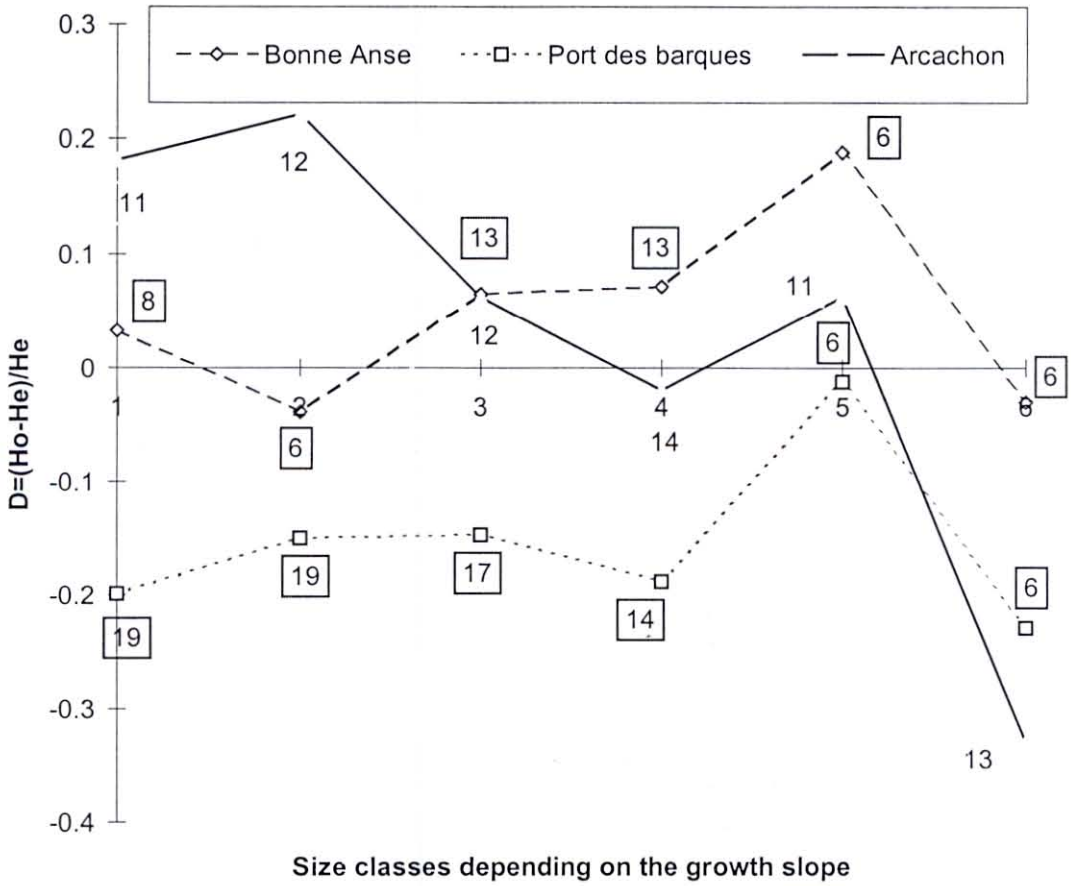

Figure 4: Relationship between mean growth slope classes and D (average over loci) in the adults sample. The number for each growth classes are outline with the population colour. The growth slope classes were : <.240, <.320, <.360, <.400, >.400 for the Bonne Anse popultion ; <.160 ,<.220, <.280, <.340, <.4, >.4 for the Port des barques population ; <.05, <.100,<.150, <.20,<.250,>.250 for the Arcachon population.

4 Discussion

In the present study, the genetic transmission between the parents and the adults showed a strong deviation from Mendelian expectations in the three populations as noted for the G1 juveniles (2nd progress report, 1997). The microsatellite markers used in the G1 juveniles also showed a strong desequilibrium in the parent contribution. For the three populations, only few families were successful (17,15, 15 in Arcachon, Bonne Anse, Port des Barques respectively) whereas in a 5*5 cross, 25 families were expected. A strong disequilibrium was shown in the 5*5 cross even though the concentration of spermatozoa and oocytes was the same in each cross. Some egg-sperm combinations produce no spat in *Crassostrea gigas* (Lannan, 1980). This may be due either to a gametic imcompatibility or a difference of spawning conditions

The relationship between heterozygosity and growth was investigated. Two negative correlations were found between heterozygosity and weight. A negative correlation between growth parameters is quite unusual in bivalve studies. In studies where a correlation between heterozygosity and growth is found, it was usually positive (See Zouros, 1987). In a laboratory cross with few parents, the correlation between

Task 7 : Research of chromosomal markers

1. Introduction

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 easily 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.

2. Materials and Methods, Results

The largest *C.gigas* cosmid genomic DNA library produced in the second reporting (i.e. containing 240,000 clones with inserts ranging in length from 6 kbp to 50 kbp) was examined as a potential source of *C. gigas* chromosome specific markers. Twenty-eight cosmid clones were chosen at random and cosmid DNA preparations were performed. After agarose gel electrophoretic analysis and OD spectophotometry to determine DNA quality and quantity, the cosmid DNA clones were digested with restriction endonucleases and analysed by agarose gel electrophoresis to determine DNA insert length. The results of this analysis are shown in table 1. The length of DNA inserts among the 28 cosmid DNA clones ranged from 10 kbp to 50 kbp (table 1).

All 28 cosmid DNA preparations were labelled by nick translation for subsequent fluorescent *in situ* hybridisation (FISH) on *C. gigas* chromosomes. Nineteen cosmid clones were labelled using digoxigenin, and ten cosmid DNA clones were labelled with biotin (table 1). All labelled cosmid DNAs were first examined by agarose gel electrophoresis to determined the lengths of the labelled DNA fragments. The lengths of the labelled DNA probes ranged from 100 bp to 700 bp (table 9).

The results of the FISH analysis of *C. gigas* chromosomes showed that twenty-three labelled cosmid clones produced no chromosome specific hybridisation signals. Instead, low-density hybridisation signals were seen scattered among all the oyster chromosomes, presumably due to the presence of repetitive DNA elements found within the cosmid DNA probes. Figure 1 shows a representative example of such non-specific FISH hybridisations. Four cosmid DNA clones (*Cg* 5, *Cg* 8, *Cg* 15 and *Cg* 26) showed far less scattering of hybridisation signals among the *C. gigas* chromosomes while recognising one chromosome pair in a more intense fashion. It may be possible to optimise the DNA labelling of these four cosmid DNA clones to produce chromosome specific markers. One cosmid DNA probe (*Cg* 1) did produce a FISH signal which clearly marked one *C. gigas* chromosome pair (Figure 2).

3. Conclusion

In summary, along with the *C. gigas* satellite element previous described by this group and with cosmid *Cg* 1, currently it is only possible to identify three of the ten *C. gigas* chromosome pairs using FISH analysis. The majority of cosmid clones examined to date do not produce chromosome specific hybridisation signals. To analyse this problem, *C. gigas* cDNA clones (i.e. copies of mRNA which do not contain repetitive

heterozygosity and fitness traits is usually not found (Beaumont, 1985 ; Foltz and Chatry, 1986 ; Mallet, 1986 ; Dillon, 1988 ; Beaumont, 1991). In the Bonne Anse adult sample, the only significant correlation was found for the Initial weight and Heterozygosity. The correlation with Heterozygosity was more consistent in the Arcachon population because it was present for the Growth slope and for the Final weight. But both correlations were negative. Negative correlation between heterozygosity and shell length in bivalves was also found in 4 months old *Mytilus edulis* (Diehl & Koehn, 1985), a result that was attributed to differential mortality with different degrees of heterozygosity.

The difference between the sub-samples in the Arcachon population may be due to an environmental difference. Some published studies have showed that the correlation between heterozygosity and growth was apparent only when individuals were ongrown under stress conditions (Scott & Koehn, 1990 ; Diehl & Koehn, 1985 ; Green, 1983 ; Rodhouse & Gaffney, 1984).The different performances (e.g., growth) between heterozygous versus homozygous individuals are expected to be both less apparent and less adaptively important in environments that minimise energy demands on metabolism (Scott & Koehn, 1990). This would include high food ratio and/ or low stress demand. The ongrowing conditions between both nurseries, Bouin and La Tremblade might be different so this would explain the correlation found in the Bouin sub-sample but not in La Tremblade sub-sample for the Arcachon populations. No great differences in heterozygote deficiency were found between juveniles and parents. A decrease of heterozygote deficiency was shown in the adults above all in the Port des Barques and the Bonne Anse populations. This kind of pattern is usually found in bivalve molluscs (See Zouros and Foltz, 1984). The Lap locus in Port des Barques was an exception. A large deficit in heterozygotes was found in the adult but not in the juvenile samples. Selection might have acted on this locus. Some studies on bivalves have already shown that some Lap genotypes can be associated with salinity acclimatisation (Koehn, 1980 ; Moore, 1980 ; Gartwaithe 1989) and spawning (Hilbish & Zimmermann, 1988) in *Mytilus edulis.*

5. References

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DNA sequences) will be labelled and examined by FISH analysis. This may provide a better source of chromosome specific markers, and will certainly determine if the problem of non-specific hybridisation is due to the presence of repetitive DNA elements within the DNA probes. Along with this approach, further cosmid DNA clones will also be examined with the aim of identifying *C. gigas* chromosome specific markers.

Significant difficulties or delay experienced during the third reporting period

The major delay currently with this task is the identification of chromosome-specific cosmid DNA clones. Of 28 cosmid DNA clones which has been tested, only one cosmid DNA clone produced a chromosome specific signal. Four other cosmid DNA clones may potentially produce chromosome specific signals after further optimisation of the DNA probe preparation technique. The majority of cosmid DNA clones resulted in very many hybridisation signals scattered throughout all the *C. gigas* chromosomes. This is probably a result due to the presence of repetitive DNA sequences within these cosmid DNA clones. In an attempt to solve this problem, DNA probes derived from *C. gigas* cDNA clones (i.e. by definition do not contain repetitive DNA elements) will be examined.

Task 9: General organisation of the project: Partners meetings

Partner 04 attended the third partners meetings in Galway (November 2nd -3rd, 1998). Details of the research progress were presented to the partners.

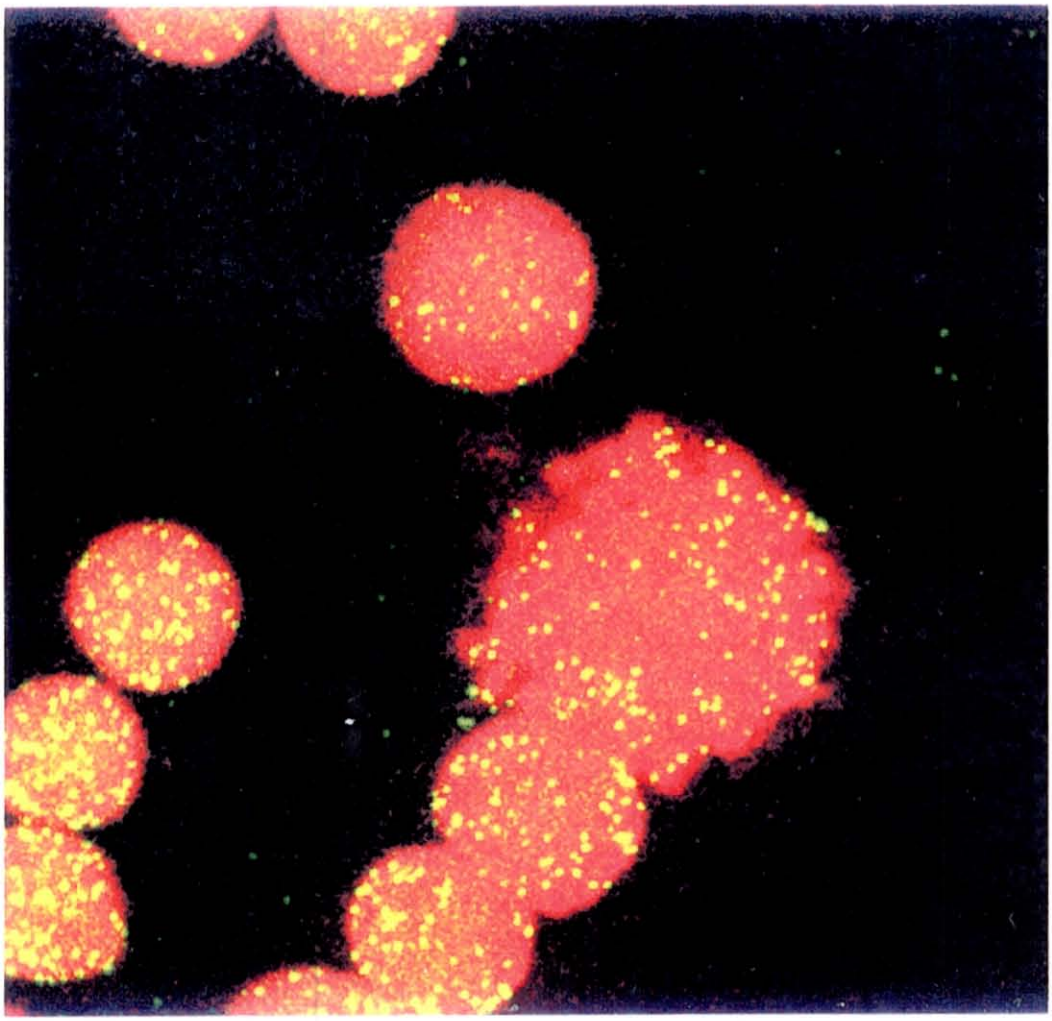

Figure 1 non-specific FISH hybridisations

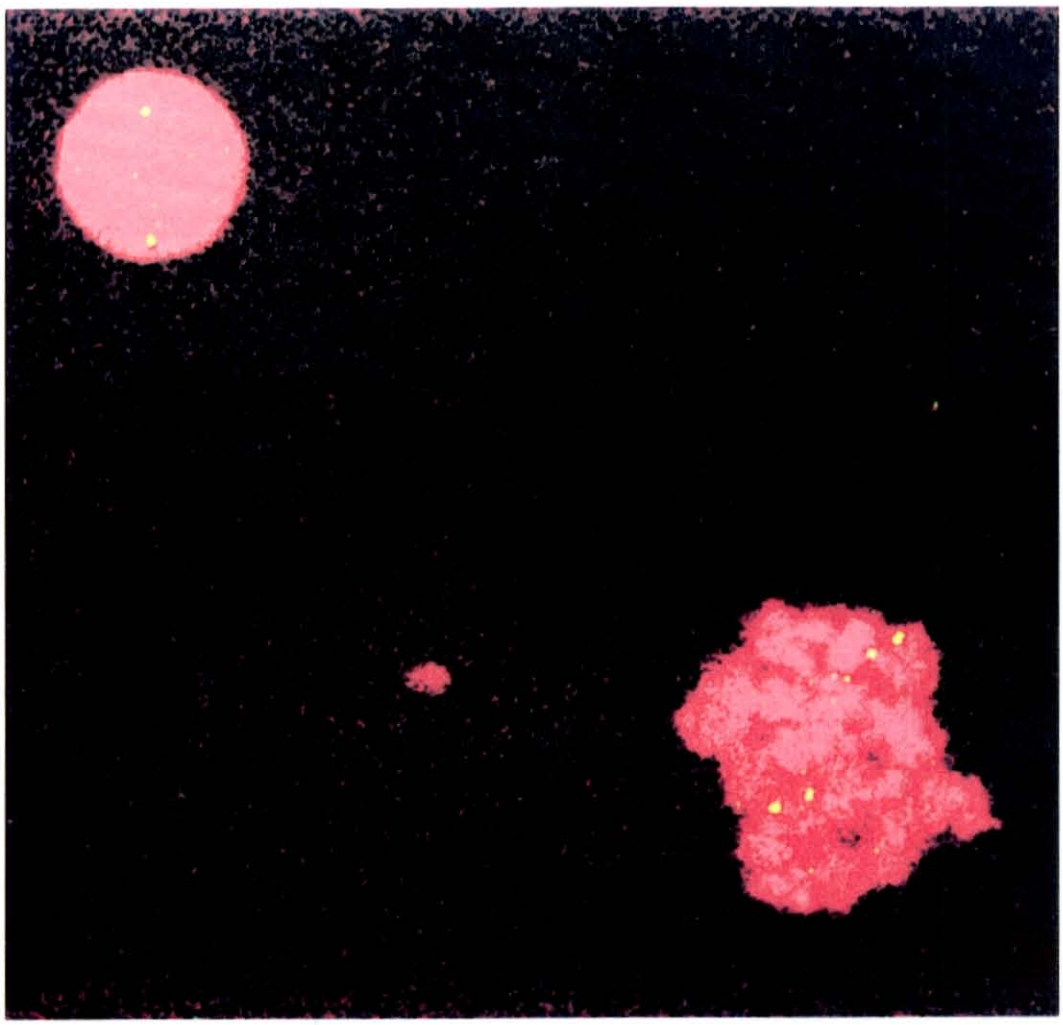

Figure 2. FISH signal which clearly marks one C. gigas chromosome pair

Cosmid No.	Probe Size	DNA Size	Label	Chromosome Specific
1	200-400 bp	24 Kbp	Digoxigenin	Yes
2	250-350 bp	17Kbp	Digoxigenin	No
3	100-250 bp	15 Kbp	Biotin	No
4	400-700 bp	20 Kbp	Digoxigenin	No
5	250-450 bp	22 Kbp	Digoxigenin	Possible
6	200-300 bp	46 Kbp	Biotin	No
7	200-400 bp	41 Kbp	Biotin	No
8	150-350 bp	39 Kbp	Biotin	Possible
9	300-500 bp	51 Kbp	Biotin	No
10	100-200 bp	40 Kbp	Digoxigenin	No
11	150-200 bp	33 Kbp	Digoxigenin	No
12	200-250 bp	19 Kbp	Biotin	No
13	450-600 bp	27 Kbp	Digoxigenin	No
14	100-500 bp	20 Kbp	Biotin	No
15	200-300 bp	44 Kbp	Digoxigenin	Possible
16	100-200 bp	30 Kbp	Digoxigenin	No
17	300-400 bp	12 Kbp	Digoxigenin	No
18	200-400 bp	23 Kbp	Digoxigenin	No
19	100-300 bp	20 Kbp	Digoxigenin	No
20	250-350 bp	18 Kbp	Biotin	No
21	200-500 bp	28 Kbp	Digoxigenin	No
22	250-400 bp	32 Kbp	Digoxigenin	No
23	300-600 bp	34 Kbp	Biotin	No
24	100-400 bp	10 Kbp	Biotin	No
25	250-300 bp	20 Kbp	Digoxigenin	No
26	200-450 bp	37 Kbp	Digoxigenin	Possible
27	300-500 bp	46 Kbp	Digoxigenin	No
28	150-300 bp	40 Kbp	Digoxigenin	No

Table 1 : Cosmid DNA clone analysis.

Annex: Task 7 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 200 ul 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* 3AI 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 66 ul 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 (FISH) analysis - DNA probe preparation.
The probe mixture consisted of 60-180 ng of labelled DNA and 12ml of 5mg/ml of boiled sheared herring sperm DNA (Boehringer Mannheim, GmbH, Germany). To this 1/10 volume of 3M sodium acetate was added. The mixture was precipitated by addition of 2.5 volumes ice cold 100% ethanol and incubated at -70°C for 4 hours. The suspension was centrifuged at 11,600 g for 15 min, and the pellet was washed by addition of 300ml of 70% ice cold ethanol and centrifuged at 11,600 g for 15 min. The pellet was the vacuum dried. Once the pellet was dried, 10ml de-ionised 50% formamide was added. The pellet was gently resuspended and vortexed. This was followed by addition of 10ml 2 X hybridization (20% w/v dextran sulphate (Sigma Chemical Co., St Louis, England), 3X SSC, 0.2% Tween 20 (Stratagene, La Jolla, CA). Again the mixture was gently pipetted and vortexed. The probe mixture was then denatured by incubating at 80°C for 6 min in a heat block. The denatured probe was then placed on ice until use.

(10) FISH analysis - Denaturation of chromosomal DNA.
Chromosome spreads were matured for 24h at room temperature, pre-treated with RNase (100µg/ml) for 1h and then dehydrated in a 70, 80, and 100% ethanol series for 2 min each. The chromosomes were denatured in 72°C in 70% formamide (v/v) for 2 min. The slides were immediately transferred to ice cold 70% ethanol for 2 min. The slides were then dehydrated in an ice cold ethanol series (80%, 95%, 100%) for 2 min each and air dried. Following this the slides and the coverslips (22mm X 22mm) were prewarmed at 37°C on a flat bed slide warmer. Next 20ul of the probe was added to the coverslip and the slide was then placed over the coverslip. Rubber cement (Sanford Corporation, Bellwood, IL 60104) was placed along the edges of the coverslip so as to seal them and the slides were incubated overnight in a humid chamber at 37°C.

(11) FISH analysis - Post-hybridisation wash procedures.
Post-hybridization washes needed to be carried out to remove unincorporated probe from the slides. The first post-hybridization wash was performed by removal of cover slip in solution A (2XSSC, 50% formamide, 0.1% Tween-20). Then the slide was washed three times in solution A at 45°C for 5 min each, followed by one wash in solution B (2xSSC, 0.1% Tween-20) at 45°C for 5 min, once in PN buffer (0.1M Na_2 HPO_4/NaH_2 PO_4 (96:3:3:7),0.1% Nonidet-P40) at 45°C for 5 min. This was followed by incubation in PNM buffer (PN buffer, 5% non-fat dry milk,0.1% Na-azide incubated overnight at 37°C) at 37°C for 15 min. This was then proceeded with the detection of the signals.

(12) FISH analysis - Immunofluorescece detection of reporter molecules.
For detection of either biotin or digoxigenin two options were used depending whether it was biotin or digoxigenin that was used. All antibodies used for detection of either hapten molecule was diluted with PNM buffer.
Biotin labelled cosmids were detected by addition of 100ml of strepavidin FITC (fluorescin isothiocyanate) (a 1:100 dilution of 1mg/ml) and incubated for 30 min in a

dark chamber at 37°C. The slides were then washed twice in PN buffer for 5 min at room temp, followed by incubation in PNM buffer at room temp for 5 min. This was then followed by a layer of 100ml of biotin labelled anti-strepavidin (1:100 of 1mg/ml) and incubated for 30 min in a dark chamber at 37°C. again washed twice with PN buffer for 5 min at room temp and incubated in PNM buffer at room temp for 5 min. This was then followed again by 100ml of strepavidin FITC (1:100 of 1mg/ml) and incubated for 30 min at 37°C. The slides were then washed twice in PN buffer for 5 min at room temp followed by incubation in PNM buffer at room temp for 15 min. Chromosomes were counterstained with propidium iodide (1ng/ml; Sigma Chemical Co., St. Louis, MO).

Digoxigenin labelled cosmids were detected by the addition of 100ml of 1mg/ml monoclonal mouse anti-digoxigenin(1:10; Boehringer Mannheim, GmbH, Germany). The slides were incubated at 37°C for 30 min. The slides were then washed twice in PN buffer at room temperature for 5 min, followed by incubation in PNM buffer for 5 min. For the second layer of antibody, 100ml of rabbit anti-mouse FITC (1:1000; Sigma Chemical Co., St. Louis, MO) was added and the slides incubated at 37°C for 30 min. The slides were again washed twice in PN buffer at room temperature followed by incubation in PNM buffer for 5 min. A final layer of antibody was then added, 100ml goat anti-rabbit FITC (1:375; Sigma Chemical Co., St. Louis, MO). The slides were again washed twice in PN buffer for 5 min and then mounted with the counterstain propidium iodide (1ng/ml; Sigma Chemical Co., St. Louis, MO).

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 1998

Partner n°5

IMBC
Institute of Marine Biology of Crete

Genetics Department

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual Progress Report
Participant n°5

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 1998**

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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ANNEX III
Individual Progress Report
Participant n°5

FAIR PL. 95.421
Individual Progress Report

For the period from
1st January to 31st December 1998

Participant n° 5: Institute of Marine Biology of Crete, Genetics Department, GREECE

Scientific team: Dr. Georgios Kotoulas
Dr. Antonios Magoulas
Mrs Aikaterini Ekonomaki
Mr. Stylianos Darivianakis
Prof. Eleftherios Zouros

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 and results synthesis Data processing, participation in result synthesis and writing of the intermediary reports and final synthesis.
• **Task 9**	General organisation of the project Participation in annual scheduled 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/ PR		FR
8		Data processing/ PR	Data processing/ PR	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 = Periodic Progress Report

FR = Final Report

Research activities during the third reporting period

Task 5 Genetical analyses

Sub-task 5.2 Microsatellites in juveniles and adults

1 Introduction

In the present study, we utilised 3 polymorphic microsatellite loci, in order to identify parentage of ; 1) 68 (G1) offspring that had been utilised in the non-destructive physiology experiment (June 97), and 2) 181 individuals (G1) of the Bouin-Growth experiment. All these animals were originally obtained by 5x5 intra-population crosses (sub-task 1.2), within animals (G0) of three different regions (Arcachon, Bonne-Anse and Port des Barques).
The genotypes of the parents were identified last year (see Second progress report), so genotyping of the above described progeny for three loci (Di10Cg, Cg49 and Cg108), was sufficient for unambiguous parentage identification.

2 Results

2.1 Gametic contribution of G0 parents to the Physiology (June 97) sample.
In table 1, the contribution of each pair of parents to the G1 generation is given. The same pattern as that for the Juvenile analysis (see Second Progress Report, 1997), is also observed here: an initial balanced gametic contribution of each parent before fertilisation, becomes an unbalanced parental contribution in the juvenile progeny.

Despite this unequal contribution of different parents, there remains a large genetic base in these animals, and therefore a large genetic variability for the characters influencing different parameters of physiology should exist. Indeed, a very first comparison of the family structure of the progeny with the physiology patterns for amylase and laminarinase, seems especially interesting, as all the members of the "physiology groups" share the same mother. Further analysis and exploitation of the combined set of data, seems very promising for the understanding the genetic and physiological basis of differences in performance.

Female/Male	B1	B2	B4	B5	J1	J2	J3	J4	J5	V1	V2	V3	V4	V5	Total
B1		1	1												2
B2			1												1
B3															
B4	2		2												4
B5		11	5	1											17
J1						1	2	1							4
J2								1	1						2
J3															
J4					1	1	5	4	3						14
J5									3						3
V1										1	1	2			4
V2												4	1		5
V3															
V4											1	4			5
V5										2		3	1	1	7
Total	2	12	9	1	1	2	7	6	7	3	2	13	2	1	68

Table 1 Parental contribution in the G1 juveniles utilised for the June-97 physiology experiment. These animals represent the G1 of the three 5x5 crosses (B stands for Port des Barques, J for Archachon and V for Bonne-Anse).

2.2 Gametic contribution of G0 parents to the Bouin-growth experiment sample.

In table 2, the contribution of each pair of parents to the G1 is given. The same comments, concerning the parental contribution, also apply here. The unbalanced contribution is very pronounced in the case of the Bonne-Anse sample, where the Female B_5 is a parent of 60/81 individuals, the Male B_2 of 48/81, and the family created by these two includes 27/81 individuals.

With further analysis, despite the unbalanced scheme, we could estimate the heritability of growth and obtain estimates of breeding values for each individual. These values will permit the definition of an optimal selection scheme for growth.

We also report here that among 181 individuals analysed one individual from the Arcachon Cross is a spontaneous triploid (oyster no. 4412). The diploid genome comes from the mother J1, and the haploid from the father J3. This animal has the 15th best performance among 82 animals of the same feeding regime and grows

twice as fast as the two other animals belonging to the same family (oysters no. 4009 and 4214).

Female/Male	B1	B2	B3	B4	B5	J1	J2	J3	J4	J5	V1	V2	V3	V4	V5	Total
B1		5														5
B2		5		2												7
B3		4														4
B4		7		2	1											10
B5	9	27	4	17	3											60
J1							1	3	2	2						8
J2						3	1	3	7	7						21
J3																0
J4						1				1						2
J5						1			1	1						3
V1													1		1	2
V2												1	14		2	17
V3													2		3	5
V4											4	1	10		1	16
V5											3		6	4	3	16
Total	9	48	4	21	4	5	2	6	10	11	7	2	33	4	10	176

Table 2. Parental contribution to the growth experiment in Bouin. These animals represent the G1 of the three 5x5 crosses (B stands for Port des Barques, J for Archachon and V for Bonne-Anse).

Task 8 Data processing and results synthesis

The main objective of the microsatellite work, that is parentage identification of progeny, has been fully achieved in this set of data also.

1) The use of only 3 (and for the big majority of only 2) highly polymorphic microsatellite loci has been very efficient in parentage identification as well as in detecting triploids and contaminants.
2) The contribution of male and female parents is unbalanced. Some animals do not contribute at all in the next generation, and others contribute the majority of the progeny. Nevertheless, we can consider that there is still a large genetic base and potential to express differences in physiological and growth performances. The most interesting information will come from the combined analysis and synthesis of all the data sets, which seems to promise a deep understanding of the variables determining differences in growth performance in *Crassostrea gigas*.

Significant difficulties or delays experienced during the third 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 1998

Partner n°6

CNRS

Centre National de la Recherche Scientifique

Laboratoire Génome et Populations, Montpellier

Reporting Period:
1st January-31st December 1998

ANNEX III
Individual Progress Report
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 1998**

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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Ass. Contractor to the coordinator

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FAIR 95-421

Individual progress Report

For the period from

1st January to 31st December 1998

Participant n° 6 **CNRS URA-1493**
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Scientific team **Dr François BONHOMME**
Jean Jacques VERSINI
Florence CORNETTE (CDD)
Véronique HERVOUET (DEA Student)

In collaboration with partner 1:
Pierre BOUDRY
Bertrand COLLET (PhD Student)

Objectives

- **Development of microsatelite markers**
- **Utilisation of microsatelite markers on larvae**
- **Data exploitation and synthesis**

Actions in the project

Task 5	Genetic analyses
Sub-task 5.3	Microsatelites in larvae Collaboration with partner 1 for the perfection of extraction and amplification of DNA from larvae and the application of microsatelite techniques to study on larvae
Task 6	Development of new microsatelite markers
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 scheduled meetings

Planned Research activities

Task	Year 1	Year 2	Year 3	Year 4	Year 5
5.3	Development of the technique/PR	Development nd application of the technique/PR	Lab analyses/PR		FR
6	Researching new microsatelite markers/PR	Research and application/PR	Lab analyses/PR	Lab analyses/PR	FR
8		Data processing/PR	Data processing/PR	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 = Progress report
FR = Final report

Research activities during the third reporting period

Task 5 Genetic Analyses

Sub-task 5.3 Microsatellites on larvae

1. Introduction

Parental contributions were analysed at different larval and spat stages in the offspring of controlled crosses of *Crassostrea gigas*, with genitors of known genotypes for a microsatelite marker. The possible effect of the gametic competition on the parental contribution to the following generation (selection or preferential mating) was evaluated by the comparison of 2 types of crosses : with gametic competition (fertilisation en masse) and without gametic competition (separate fertilisations). The analysis of different larval and post larval stages also allowed the study of evolution of relative parental contribution through time, and the detection of any differential mortality between families.

2. Materials and Methods

2.1 Crosses

One hundred oysters were sampled from the natural environment (French Atlantic coast) and labelled individually. These were matured 6 weeks at the La Tremblade hatchery before DNA testing. Biopsies were performed on the animals and DNA extracted by the Phenol-Chloroform method.
From the 100, 5 males and 5 females were chosen with different microsatelite sequences at the L10 locus in order to have parents with identifiable offspring. The parents were chosen to be heterozygote at L10 and to have no shared alleles in order to ease the task of parental analysis in the next generation and avoid ambiguity caused

by nul alleles (Koorey et al 1993; Callen et al 1993).
The 5 males and 5 females were crossed in two different ways :

Cross S : 25 separate crosses. 25 separate fertilisations in beakers – mixed together 3 hours later.

Cross M : A mixed cross. Fertilisation of the 5 females by the 5 males in a sole beaker.

In both cases the sperm: ovule ratio was controlled at 150:1. The two larval populations from these crosses were each divided into 3 replicate blocks. At the age of 6 days (S + M crosses) and 18 days (S cross) the larvae were separated, measured to 1µm (with a Nikon profile projector) before parental analysis was performed. At 90 days, samples of post-larvae were separated, weighed to 0.01g and analysed to identify their parents.

2.2 Microsatelite analysis

Microsatelite analysis was made on larval DNA extracted by the Chelex method (Walsh et al 1991; Bierne et al 1998; Schizas et al 1997, see Annex 1) PCR amplification was done using primers produced by partner 5 and partner 6. Size of PCR products, temperatures and $MgCl_2$ concentrations are indicated in table 1.
PCR products were migrated on polyacrylamide gel for 2 ½ hours. The gel was then dried for 2 hours and put with a Kodak BioMax film for 6-48 h depending upon the level of radioactivity.

Locus	Tm (°C)	Pattern	Primer (S: sense A: Antisense)	Size of cloned fragment
L10	55	$(AG)_n$	S: cat gtt ttc cct tga ctg atc c	330
			A: ggt caa ttc aaa gtc aat ttc cc	

Table 1 Primers used for the L10 locus. Tm =Optimal pairing temperature

2.3 Data analysis

We developed a Visual Basic program to identify the possible parents for any one genotype and one locus. A G test (Sokal and Rohlf 1995) was used to calculate the different parental contributions for : a single type of cross, a single stage of development, between the 5 males and 5 females, between the 25 families, between the 2 types of cross (M + S), between 2 stages of development (D6, D18 and D90) and between blocks of a single type of cross.
To evaluate effects of male–female interaction for each sampling, i.e. heterogeneity resulting from preferential mating, an overall value, Gg was calculated. This is the sum of the G values of the parental contributions:

- for the 5 males with each of the 5 females
- for the 5 females with each of the 5 males

This gives 10 G_i values. A residual G_r, corresponding to male-female interaction is calculated from :
$G_r = \Sigma G_i - G_f = G_g - G_f$, where G_f measures the heterogeneity between families.

Size and weight differences were analysed by ANOVA (SAS PROC GLM). Segregation at locus L10 was analysed by a X^2 chi test with 1:1 frequencies as the expected frequencies.

3. Results

3.1 Separate and Mixed crosses

At D6, 272 and 249 individuals were analysed respectively for the separate cross (S) and for the mixed cross (M). At D18, 399 individuals were analysed for the S cross. After fixation at D90, 244 individuals were analysed for the S cross. The parental analysis was conducted easily because almost all 10 chosen parents had different alleles at the L10 locus, and were heterozygote for this locus (parental genotypes in table 2). Only M1 and F1 parents share a common allele (25), which doesn't matter as these 2 individuals are the only ones who do. In the offspring the 19 alleles segregated and no new alleles, that were not present in the parents, appeared. This confirms that no mutation or zootechnical contamination occurred in the studied samples.

	Gametic segregation				Gametic segregation		
Parents	**Allele**	**Nb**	**P**	**Parents**	**Allele**	**Nb**	**P**
M1	18	159	0.128	F1	25	122	0.002
	25	133			31	78	
M2	14	35	< 0.0001	F2	12	95	0.012
	28	138			30	133	
M3	19	62	< 0.0001	F3	11	125	< 0.0001
	23	150			17	73	
M4	-2	121	0.027	F4	21	162	< 0.0001
	15	158			24	32	
M5	9	88	0.019	F5	26	132	< 0.0001
	34	122			37	214	

Table 2 Parental genotypes and gametic segregation at locus L10 for the 10 parents. P is the probability of the observed X2 frequency against the expected X2 frequency (1:1 segregation)

3.2 Segregation at the L10 locus

The analysis of the gametic segregation at L10 locus, both crosses together, shows a significant distortion of segregation for all the parents apart from the M1 male (table 2). The analysis of the zygotic segregation per family is presented in annex 2.

For each parent, the frequency per allele does not vary between D6 and D18 for the S cross. So, the result from these 2 stages were put together (referred to as D6-D18 S). Between D6-D18 S and D90 S, the frequency per allele does not change significantly apart from the F4 female (P=0,0194) : the segregation is 64:22 at D6-D18 S and 80:6 at D90 S, the difference between the 2 alleles increases over the course of time. The frequencies per allele are not significantly different at D6 between the two types of cross S and M.

The same type of result is shown on table 3 for each cross and stage of development.

The gametic segregation of the M1 male is only significantly unbalanced for the D6 stage (P=0,04).

3.3 Parental contributions

For each sampling point, there is no significant difference of parental contributions between the 3 blocks, which allows the merging of this date (table 4). The comparison between the observed and expected results, in which each of the 5 males and 5 females would have contributed in an equal way, shows that there is a large imbalance of parental contribution (table 5).

Only the mixed cross at D6 presents a significant difference of contribution between the 25 families (P=0,006). There is no significant heterogeneity due to the interaction between male and female whatever the sampling date (table 6). Nevertheless, at D18, the family 21, corresponding to the male 1 x female 5 cross, is clearly more strongly represented than the others. In fact, the disparities shown on the graphs are essentially due to differences between male contributions, female contributions, or to their combined effects.

Gametic Segregation

		D6-D18 S		D90 S		D6 M			
Parent	**Allele**	**Nb**	**p**	**Nb**	**p**	**Nb**	**p**	**D6-D18S vs D90S**	**D6S vs D6 M**
M1	18	105	0.835	31	0.090	23	0.063	NS	NS
	25	102		19		12			
M2	14	18	6,E-11	10	0.239	7	<0.0001	NS	NS
	28	84		16		38			
M3	19	36	0.005	15	<0.0001	11	0.004	NS	NS
	23	64		57		29			
M4	-2	73	0.934	8	0.001	40	0.084	NS	NS
	15	72		29		57			
M5	9	41	0.002	32	0.515	15	0.398	NS	NS
	34	75		27		20			
F1	25	80	0.006	21	0.077	21	0.631	NS	NS
	31	49		11		18			
F2	12	64	0.072	11	0.827	20	0.024	NS	NS
	30	86		10		37			
F3	11	60	0.020	44	0.003	21	0.411	NS	NS
	17	37		20		16			
F4	21	64	6,E-06	80	<0.0001	18	0.003	0.0194	NS
	24	22		6		4			
F5	26	81	0.001	15	0.086	36	0.011	NS	NS
	37	127		26		61			

Table 3 Parental genotypes and gametic segregation at locus L10 for the 10 parents and for the separate cross (S) at D6 and D18 together and D90 and for the mixed cross (M) at D6. P is the probability of the observed X^2 frequency against the expected X2 frequency (1:1 segregation). D6-D18S vs D90S is the comparison between D6-D18S and D90S. D6S vs D6M is the comparison between the separate and mixed crosses at D6. NS= Non significant at the 5% level.

			Males		Females	
Date	Cross	Comparison	G	p	G	p
D6	mixed	1-2	2.27	0.686	0.85	0.931
		1-3	4.03	0.402	1.11	0.893
		2-3	5.53	0.237	4.54	0.337
D18	separate	4-5	2.03	0.73	1.34	0.854
		4-6	7.86	0.097	3.99	0.407
		5-6	7.49	0.112	9.41	0.052
D18	mixed	1-2	1.47	0.832	1.1	0.895
		1-3	3.04	0.551	1.02	0.907
		2-3	2.09	0.72	0.76	0.943

Table 4 Comparison of parental contributions between the replicates (1 to 3: separate cross) (4 to 6: mixed cross)

A.

D6 S	F1	F2	F3	F4	F5	%
M1	18	10	8	9	25	*0.26*
M2	9	13	11	8	19	*0.22*
M3	8	8	3	10	14	*0.16*
M4	11	17	4	5	21	*0.21*
M5	10	13	7	6	5	*0.15*
%	*0.21*	*0.22*	*0.12*	*0.14*	*0.31*	**1**

Males		Females	
G	p	G	p
2.81	0.589	7.57	0.109

B.

D6 M	F1	F2	F3	F4	F5	%
M1	4	1	3	0	26	*0.14*
M2	7	8	5	5	21	*0.18*
M3	4	14	2	9	11	*0.16*
M4	19	21	20	4	31	*0.38*
M5	5	9	6	6	8	*0.14*
%	*0.16*	*0.21*	*0.14*	*0.1*	*0.39*	**1**

Males		Females	
G	p	G	p
10.24	0.037	13.95	0.007

C.

D18 S	F1	F2	F3	F4	F5	%
M1	25	20	17	19	56	*0.34*
M2	4	12	4	5	16	*0.1*
M3	8	11	10	8	17	*0.14*
M4	20	22	17	6	22	*0.22*
M5	19	17	12	17	15	*0.2*
%	*0.19*	*0.2*	*0.15*	*0.14*	*0.32*	**1**

Males		Females	
G	p	G	p
16.81	0.002	8.807	0.066

D.

D90 S	F1	F2	F3	F4	F5	%
M1	8	2	17	17	5	*0.2*
M2	2	3	5	12	4	*0.11*
M3	11	4	16	20	21	*0.29*
M4	1	7	11	14	4	*0.15*
M5	10	5	15	24	6	*0.25*
%	*0.13*	*0.09*	*0.26*	*0.36*	*0.16*	**1**

Males		Females	
G	p	G	p
7.401	0.116	14.709	0.005

Table 5 Parental contributions: Contingency tables showing frequencies per family with overall frequencies for the different males and females, in the separate cross at D6 (A), D18 (C) and D90 (D) and the mixed (M) cross at D6 (B). The tables on the right give the G statistics and the probability of the observed frequencies against the expected frequencies (equal contributions:1/25 for each of the 25 families)

		Families		male x female interaction	
Date	Cross	G_f	p	G_r	p
D6	M	44.86	0.006	16.1	0.446
D6	S	16.44	0.872	16.1	0.446
D18	S	32.52	0.115	8.43	0.935
D90	S	31.85	0.131	6.8	0.977

Table 6 Heterogeneity between families and heterogeneity due to male x female interaction, for each sampling point.

3.4 Gametic competition

Both types of cross were analysed at D6, the larval stage soonest after fertilization which is sufficiently advanced to allow PCR amplification. Male contributions are significantly different between the types of crosses (G=11,26; P=0,024; figure 1; but female contributions are not (G=3,23; P=0,520; figure 2). M and S crosses were combined and the heterogeneity of female contributions analysed at D6: the heterogeneity between females is once more very significant (G=19,7; P=0,0006; table 7).

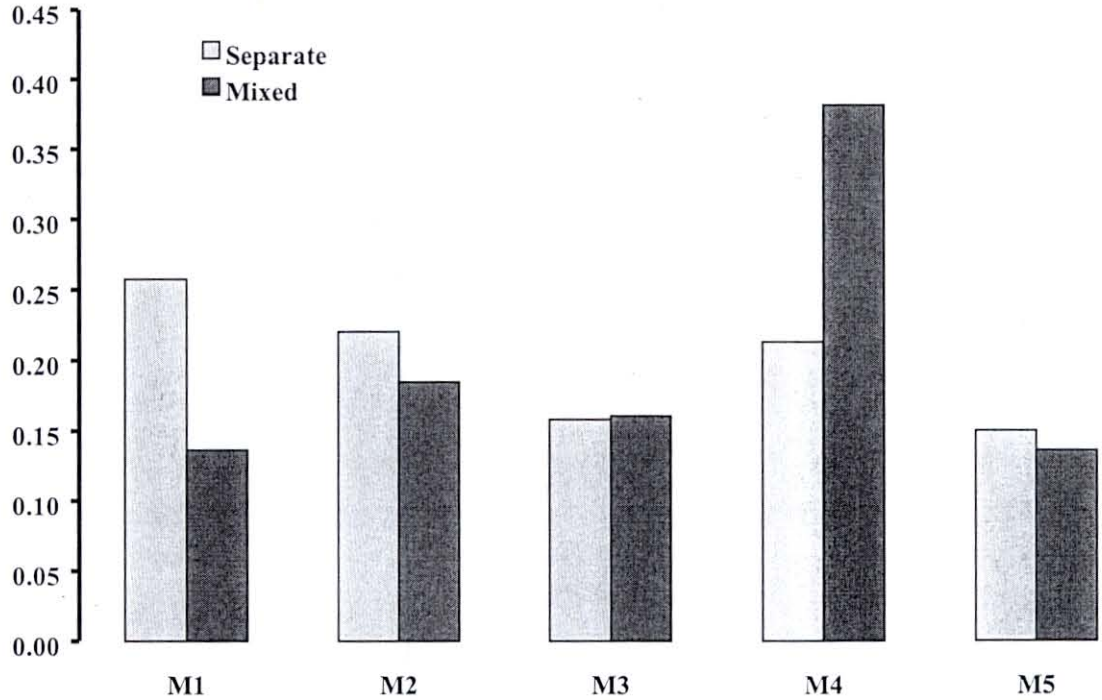

Figure 1 Contribution (frequencies) of males (M1 to M5) in the two types of crosses, separate and mixed, at D6

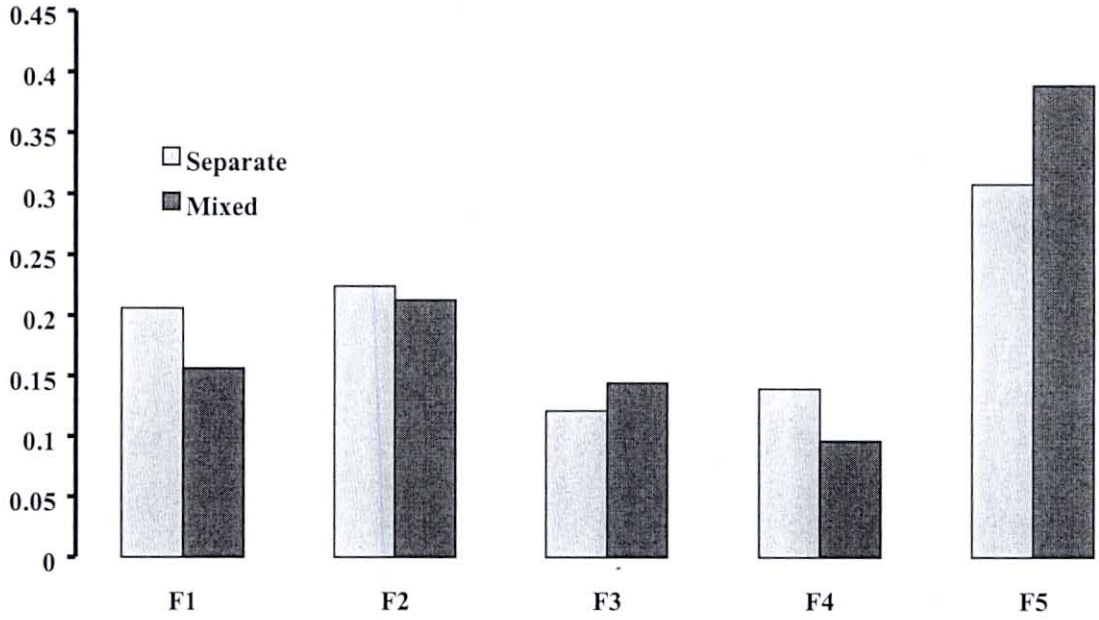

Figure 2 Contribution (frequencies) of females (F1 to F5) in the two types of crosses, separate and mixed, at D6

D6 S+M	F1	F2	F3	F4	F5	%
M1	22	11	11	9	51	*0,20*
M2	16	21	16	13	40	*0,20*
M3	12	22	5	19	25	*0,16*
M4	30	38	24	9	52	*0,29*
M5	15	22	13	12	13	*0,14*
%	*0,18*	*0,22*	*0,13*	*0,12*	*0,35*	**1**

Table 7 Female contributions in the separate (S) and mixed (M) crosses at D6

3.5 Changes in population genetic structure over time

Changes in the parental contributions over time are illustrated on figures 3 and 4, showing the incremental parental contributions in the S cross at stages D6, D18 (before metamorphosis) and D90 (3 month-old juveniles). The offspring of the crosses are presently being analysed for D18 and D90 M. Between the D6 and D18 stages, there is a significant difference in male contributions (G=11,94; P=0,018) but not in female contributions (G=0,90; P=0,925). Between the D18 and D90 stages, these contributions are significantly different for males (G=14,03; P=0,007) and for females (G=29,06; $P=7{,}596.10^{-6}$).

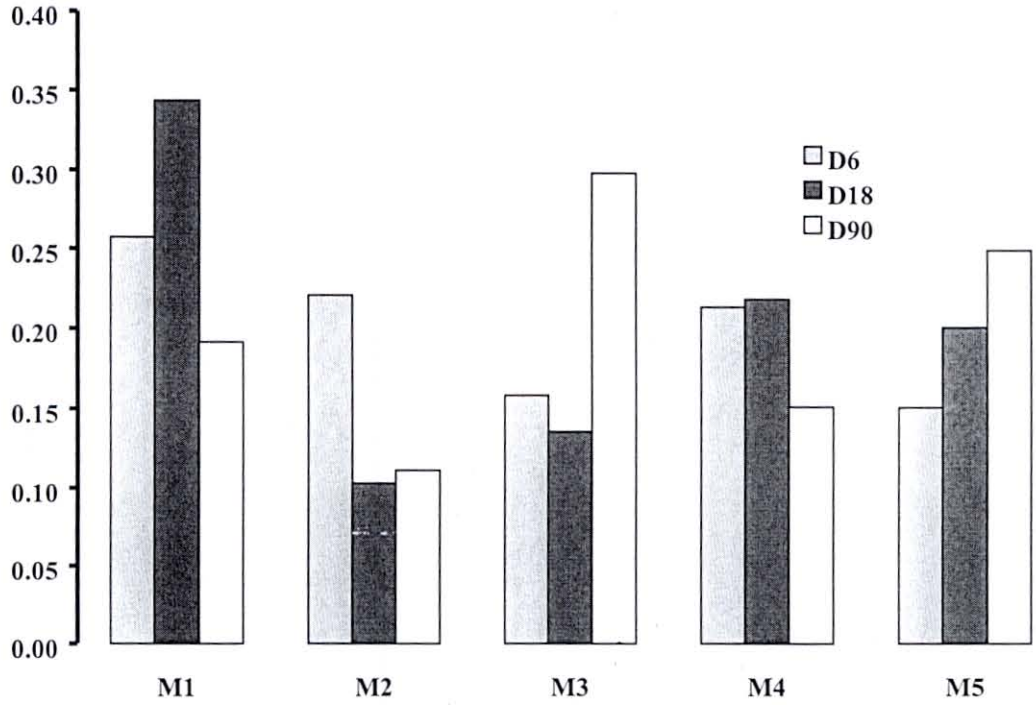

Figure 4 Contribution (frequencies) of females (F1 to F5) in the separate cross at D6, D18, and D90

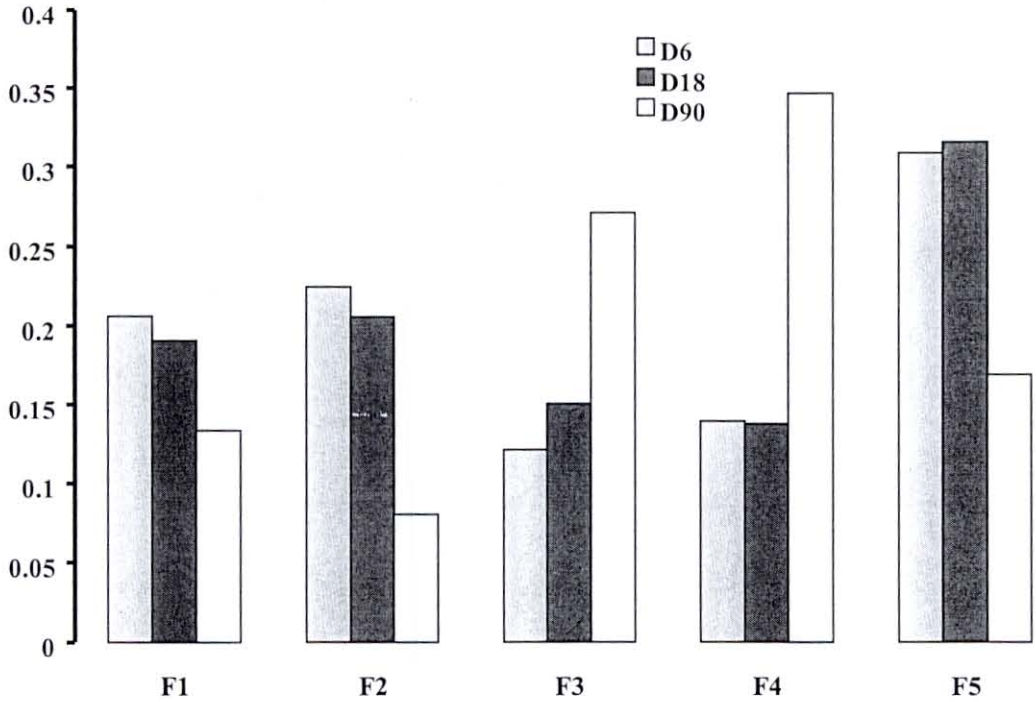

Figure 3 Contribution (frequencies) of males (M1 to M5) in the separate cross at D6, D18, and D90

3.6 Comparison of spermatic competition with male offspring survival

The comparison between the table of the parental contribution of the M cross, at D6 (table 5A) and the table which represents the parental contributions of the S cross at D18 (table 5C) allows testing of the correlation between male efficiency in the case of spermatic competition and the survival of the larval offspring of this same male. These two tables are significantly different (G=33,69; P=8,626.10^{-7}), showing that there is no correlation between contribution and survival for males.

3.7 Growth

No cross analysed at any stage, shows any significant difference in size (or weight) between either the 25 families or between the offspring of the 5 males or the 5 females. Nevertheless, significant differences of size are observed between the mixed (1,2 and 3) and separate (4,5 and 6) crosses at D6 (tables 8, 9 and 10), and between all the different batches at D6 and D18 (table 11), in the same way there is a significant weight difference between batches at D90 (table 11).

	Mixed	Separate
D6 (µm)	106.5 ± 0.49	100.65 ± 0.54
D18 (µm)		268.69 ± 2.13
D90 (g)		0.77 ± 0.29

Table 8 Size (µm) and weight (grams) means (± s.e.) of individuals at each sampling date

	Batches					
	1	2	3	4	5	6
D6 (µm)	106.61 ± 0.81	102.24 ± 1.24	108.04 ± 0.82	100.13 ± 0.65	98.78 ± 1.69	102.19 ± 0.81
D18 (µm)				274.82 ± 3.67	261.15 ± 3.64	267.21 ± 3.60
D90 (g)				0.82 ± 0.04	0.70 ± 0.03	0.78 ± 0.04

Table 9 Size (µm) means (± s.e.) of individuals in each different batch at D6 and D18.

Source of variation	d.f.	Sums of Squares	Mean squares	F	P
Model	1	4469	4468.56	67.53	0.0001
Error	515	34079	66.17		
Total	520	39887			

Table 10 Analysis of variance to test the effect of cross type (S or M) on size of offspring at D6.

Stage	Source of variation	d.f.	Sums of Squares	Mean squares	F	P
	Model	5	5808	1161.56	17.55	0.0001
D6	Error	515	34079	66.17		
	Total	520	39887			
	Model	2	11908	5953.9	3.34	0.037
D18	Error	396	706484	1784.05		
	Total	398	718391			
	Model	2	0.72	0.36	3.16	0.044
D90	Error	241	27.54	0.11		
	Total	243	28.27			

Table 11 Test of heterogeneity in length (D6 and D18) and weight (D90) between batches

4 Discussion

4.1 Unbalanced parental contributions

The study of 2 types of cross with and without gametic competition revealed very large imbalances in the contributions of the males and females. Nevertheless, it was not possible to observe any incompatibility between the gametes, all the families contributed to the formation of the following generation, even in the case of gametic competition. The imbalance of the contributions is only significant for the cross with gametic competition. In all cases, the heterogeneity within crosses is due to the differences of contribution between the 5 females and between the 5 males; the interactions between males and females, which would result from preferential fertilisations, are not a source of heterogeneity. Because the frequency in each family is often low, the heterogeneity due to the interaction between males and females is not easy to estimate with precision. Differences in the state of maturation of females on one hand and differences in the sperm quality (motility in particular) of males on the other hand, can explain such imbalances in male and female contributions 6 days after fertilisation. These results imply that imbalances could be partly due to processes occurring very early in development. Such results are in accordance with the study of Li and Hedgecock (1998) on natural populations of the same species and show that reproductive success is an extremely variable character.

4.2 Spermatic competition

This experiment demonstrates the presence of spermatic competition in *Crassostrea Gigas* shown at the larval stage, 6 days after fertilisation, in controlled hatchery conditions. However, there is no competition between oocytes. This result is easily explained considering that oocytes are immobile and that spermatozoa with the best motility achieve the best fertilisation. In the separate crosses with 150 spermatozoa per oocyte, it is very likely that the fertilisation succeeded. Under conditions of gametic competition, the most active spermatozoa "saturate" the oocytes present and the blocking mechanisms of polyspermy prevent the weaker spermatozoas from contributing to the following generation.

There is no proof of polyspermatic blocking in molluscs in the strict sense (Raven, 1966) ; sometimes, many spermatozoa can enter the oocyte, but only one develops into a pronucleus (Crabb, 1927; De Laramberg, 1939; Bretschneider, 1948; Hortsmann,

1955) with all the extra male nuclei disappearing. So, the present result demonstrates the existence of gametic competition and more precisely of spermatic competition.

4.3 Evolution during the larval stage

During the larval stage, the parental contributions of males change between D6 and D18 and become significantly different. This result can only be explained by differential mortality of larvae. However, males which were favoured by the spermatic competition are not those whose larvae have the best survival. The M4 male, has many offspring at D6, but these have lower survival during the larval stage than offspring of M1 male, despite the fact that M1 larvae were not numerous at D6.

Female contributions are unbalanced, but do not change during the larval stage between D6 and D18. The maternal effect of imbalance at the beginning, shown by strong differences between female contribution at D6, is therefore not disturbed by the differential mortality between D6 and D18.

4.4 Evolution after metamorphosis

Although differences in larval survival can be seen to vary according to paternity, this variation is much greater in 3 month-old juveniles (D90). The males which contribute the most at this stage are not the same at the final larval stage. The M3 male, in particular, accounts for an increased percentage of surviving offspring at D90 even though they were relatively few at the larval stage (Figure 3). At the same time, the number of surviving M1 male offspring decreases strongly. A similar phenomenon occurs in the females : the contribution of females F3 and F4 clearly increases (for F4 it doubles) while for female F5 it falls (Figure 4).

Two non-exclusive hypotheses can be proposed to explain these changes.

- Between the two sampling stages D18 and D90, metamorphosis, a critical step in development, took place. Metamorphosis involves large physiological modifications. After metamorphosis different genes will be expressed from in the larval stage and individuals will respond differently to selection pressures by different levels of survival.
- A high death rate (up to about 90%), the cause for which has not been determined, was observed on the spat. It is possible that the response of juveniles had a genetic basis and caused differences in contributions independently of events at the larval stage.

4.5 Growth

For the first 3 months, growth based on length or weight, appears to be the same between the families. This result shows that survival to D90 is independent of growth.
Differences of size and weight observed between batches which are particularly significant at D6, are probably due to differences in density in the tanks. Batch 3, which has the highest mean size at D6 is also that with the lowest density (87,000 rather than 150,000 as in the other tanks). Densities can change rapidly from one day to the next because high mortality can occur during the larval stage. These differences lessen with time, which can be explained by the phenomenon of larval compensation growth, a greater speed of growth in small larvae allowing them to catch up with larger individuals, or alternatively by different mortality rates associated with an interaction between growth and mortality.

At D6 a highly significant difference is observed between the mixed (M) and

separate (S) crosses. This difference probably relies to some extent on differences between replicates but this doesn't explain the high level of significance ($P<0.0001$). The larvae from the mixed cross are larger at this stage, which could result from elimination of less adapted individuals (those smaller from the start, or with weaker growth) from the zygotic stage.

4.6 Changes in parental contribution over time

Of the 10 parents used, all contributed to the formation of the next generation and no preferential fertilisation was shown. At the same time, despite measures taken to equilibrate the gametic quantities of males and females at fertilisation, the parental contributions are not equal. This starts at the fertilisation or zygotic stage when competition between parents exists, which is effectively the case in the natural environment and in mass crosses in hatcheries. This phenomenon could be explained by differences in gamete quality (Lannan, 1980a, 1980b, 1980c and 1980d), which can occur because different parents mature earlier or later than others even in common environmental conditions. These differences can cause maternal and possibly also paternal effects. Contributions also vary over time, with a notable change between the larval and juvenile phases (Lannan 1980a). During the larval phase, the influence of maternal and paternal genotypes differs. For males it is primarily spermatic competition that determines their contribution, then differential survival of their offspring.
Female contributions are influenced by maternal effects which function from the zygotic stage until metamorphosis. Survival rates were estimated throughout the culture period : after fertilisation, only 30% of ovocytes attain the D-shaped larval stage, 3% survive to become pedelargic larvae and only 0.1% survive to the spat stage at 2 months.

Finally, certain individuals participate more than others in the formation of the new generation of juveniles and subsequent adults. A continuing study of offspring obtained would allow us to determine if the individuals with the largest parental contributions at the juvenile stage remained the same until the offspring were adults, or if new changes occurred. For oyster breeding, the best choice of genitors would be those which are best represented amongst the adult offspring of the next generation, taking into account the risks of exposing genetic load through inbreeding (Sheridan 1997). It remains to be verified that the experiment is repeatable and whether it is always the same genitors with the largest parental contributions under other production conditions. In the present experiment, the three replicate blocks only represent pseudo-replicates, the division having been made only three hours after fertilisation. The analysis of individuals from a cross mixed at D18 or after metamorphosis would allow the confirmation of the present results. An increased number of individuals genotyped per cross would also make the statistical tests more solid.

Certain genitors show significant segregation distortion. While these distortions don't vary at all between D6 and D18, they vary for one individual (Female F4) between D6-D18 and D90. These results show that segregation distortion appears very early for most of the genitors and is maintained over time except for female F4 where the levels of representation of 2 alleles cross due to differential mortality. Allele 24 is under represented at D90 compared with at D6-D18 showing that mortality rate is positively related to this allele.

No correlation was found between survival and growth in any family, although this doesn't mean that such a relationship might appear later in development. The analysis of parents at several microsatellite loci would allow the estimation of heterozygosity levels in each family and any relationship between these levels and

survival.

Studies on the contribution of each parent to the formation of the next generation allow us to examine the factors influencing these contributions. The use of genomic markers ensures increased experimental repeatability. This is the first time that such a study on parental research, with attribution of all the offspring to biparental families and surveying of the population through time has been made in molluscs. It allows the extension of our knowledge on life cycle events in the Pacific oyster which influence the genetic structure of a population according to the environment and the age of individuals it is made up of. Knowledge of this sort will allow rapid selection of genitors producing individuals with the highest survival and growth performances.

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6. Annexes

L10 locus, Larvae and Adults	
Primer 1	0.189µl
Kinase Buffer 10X	0.142µl
γ-^{33}P-ATP	0.0788µl
T4 polynucleotide kinase	0.0079µl
Water	1µl
Final volume	1.4177µl
Primer 2	0.189µ1
Taq Buffer 10X	1.05µl
$MgCl_2$	0.42µl
DNTP	0.39µl
Taq	0.074µl
DNA	8µl
Final volume	11.541µl

Annex 1 Reagent details of protocol for Chelex extraction of DNA from Crassostrea gigas

Annex 2. Zygotic segregation per family

		Results		
Family	zygote	Observed	Expected	P
M1 X F1	1825	16	13	0.006
	1831	7	13	
	2525	22	13	
	2531	7	13	
M1 X F2	1812	7	9.75	0.264
	1830	8	9.75	
	2512	15	9.75	
	2530	9	9.75	
M1 X F3	1811	13	11.75	8.9E-05
	1817	24	11.75	
	2511	7	11.75	
	2517	3	11.75	
M1 X F4	1821	15	10.5	0.019
	1824	4	10.5	
	2521	16	10.5	
	2524	7	10.5	
M1 X F5	1826	25	28	4.8E-08
	1837	40	28	
	2526	3	28	
	2537	44	28	

		Results		
Family	zygote	Observed	Expected	P
M2 X F1	1425	1	5.75	1.7E-04
	1431	1	5.75	
	2825	14	5.75	
	2831	7	5.75	
M2 X F2	1412	5	9.5	4.0E-04
	1430	3	9.5	
	2812	10	9.5	
	2830	20	9.5	
M2 X F3	1411	6	6	0.072
	1417	2	6	
	2811	11	6	
	2817	5	6	
M2 X F4	1421	10	7.25	4.3E-03
	1424	0	7.25	
	2821	15	7.25	
	2824	4	7.25	
M2 X F5	1426	5	14.75	8.1E-08
	1437	2	14.75	
	2826	29	14.75	
	2837	23	14.75	

		Results		
Family	zygote	Observed	Expected	P
M3 X F1	1925	5	8	0.100
	1931	4	8	
	2325	11	8	
	2331	12	8	
M3 X F2	1912	2	8.75	0.019
	1930	7	8.75	
	2312	12	8.75	
	2330	14	8.75	
M3 X F3	1911	9	8.75	8.9E-06
	1917	2	8.75	
	2311	21	8.75	
	2317	3	8.75	
M3 X F4	1921	16	11.5	4.0E-04
	1924	0	11.5	
	2321	19	11.5	
	2324	11	11.5	
M3 X F5	1926	4	16	2.6E-04
	1937	13	16	
	2326	28	16	
	2337	19	16	

		Results		
Family	zygote	Observed	Expected	P
M4 X F1	1525	12	12.5	0.270
	1531	7	12.5	
	-225	15	12.5	
	-231	16	12.5	
M4 X F2	1512	16	18.25	0.034
	1530	25	18.25	
	-212	9	18.25	
	-230	23	18.25	
M4 X F3	1511	19	13	2.5E-06
	1517	25	13	
	-211	8	13	
	-217	0	13	
M4 X F4	1521	18	7	4.0E-07
	1524	0	7	
	-221	10	7	
	-224	0	7	
M4 X F5	1526	14	19	0.015
	1537	22	19	
	-226	11	19	
	-237	29	19	

		Results		
Family	zygote	Observed	Expected	P
M5 X F1	3425	15	10.75	0.445
	3431	9	10.75	
	925	11	10.75	
	931	8	10.75	
M5 X F2	3412	9	10.75	0.152
	3430	17	10.75	
	912	10	10.75	
	930	7	10.75	
M5 X F3	3411	20	10	0.001
	3417	7	10	
	911	11	10	
	917	2	10	
M5 X F4	3421	20	12.25	2.3E-06
	3424	5	12.25	
	921	23	12.25	
	924	1	12.25	
M5 X F5	3426	5	8.75	0.090
	3437	15	8.75	
	926	8	8.75	
	937	7	8.75	

Annex 2. Zygotic segregation per family

Task 6 Development of new microsatelite markers

This task has now been effectively completed. Sufficient microsatelite markers have been identified and developed as to provide the tools necessary for the ongoing research in which they are applied (Task 5.3).

European Commission

Contract No. FAIR 95-421

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

Minutes of the annual meeting
Galway
2nd-3rd November 1998

Reporting Period:
1st January-31st December 1998

Minutes of the third GENEPHYS meeting

held in Galway the 2nd and 3rd November 1998.

Present :

Partner 1: IFREMER
J.P. Baud (Bouin), P. Boudry (La Tremblade), S. Bougrier (L'Houmeau), J.F. Samain (Brest)

Partner 2: NERC (Plymouth Marine Laboratory)
A. Day, A.J.S. Hawkins

Partner 3: CNRS (Observatoire Océanologique de Villefranche sur Mer)
A. Leitao, C. Thiriot

Partner 4: National University of Ireland, Galway
S. Hubert, R. Powell, G. Rafferty, N.P. Wilkins

Partner 5: Instute of Marine Biology of Crete

Partner 6: CNRS (Laboratoire Génome et Populations)

DG XIV:
Tarja Tiainen

1. Introduction:

Due to travel and/or health problems, 4 French participants (Partner 1), were not able to attend the meeting. Consequently, Pierre Boudry, chaired the meeting in the place of André Gérard,

The schedule was as follows:
First day : presentation of 1997-1998 results.
Second day :synthesis of the first 3 years in the presence of the representative of DG XIV (morning), discussion of experimental plans for 1999 (afternoon).

2. Progress reports

2.1. Results of the « June 97 » G1 experiment

In June 1997, 68 oysters, which had already been individually recorded for growth in La Tremblade were studied for physiological and genetic parameters. These animals were first acclimated (to low food availability and 15°C), then non destructive studies were made on physiological traits (P1b), followed by a destructive physiological study (P2) and finally animals were dissected and sampled for physiological (P1d, P2) and genetic (P4, P5) analyses.

2.1.1. Non destructive study of physiology (P1b)

The 68 oysters were studied for oxygen consumption, filtration, ingestion and assimilation rates. No significant differences were observed between the 3 « 5 x 5 » crosses. Unlike previously published results, oxygen consumption was found to be linearly (and not allometrically) linked to dry weight. The analysis of scope for growth tends to support the hypothesis of two sub-groups (positive and negative values) in the studied material.

2.1.2. Protein turn-over and proteolytic activities (P2)

Protelytic activities of cathepsin D and LAP were recorded on the same 68 oysters, using 'remainder tissues' due to transport problems with digestive, muscle and gill tissues. Activities were found to be much higher in *Crassostrea gigas* than in *Mytilus edulis*, although this is not the case for cathepsin B. All these parameters were found to be negatively allometrically related to dry weight. A new assay was developed for CANP (Calcium-dependant Protease).

Protein turn-over and net ammonium losses were recorded on 40 out of the 68 oysters (20 large + 20 small). These parameters, ten times higher in *C. gigas* than in *M. edulis*, were found to be negatively allometrically related to dry weight. Moreover, they are significantly higher in individuals which are heterozygote at the DAP locus (recorded by P4) than in homozygotes. This is not the case for lysosomal proteins.

Additionally, activities in digestive gland, gill and remaining tissues have been recorded in fast and slow growing oysters from the Bouin experiment (July 1997).

2.1.3. Digestive enzyme activities (P1d)

Within the 68 oysters, two sub-groups can be defined according to the methods described in the second progress report. New DNA makers of the amylase gene(s) were developed in 1998. Four different types within the 68 individuals can be distinguished by PCR amplification. In combining types 1-2 and 3-4, a significant difference for relative growth was observed between groups. The hypothesis would be that two different kinds of oysters can be distinguished: 'high feeders' that perform well in high but not in low food availability, and 'low feeders' that perform at a low level whatever the food availability.

2.1.4. Allozyme marker analysis (P4)

The 68 oysters were analysed for 11 loci. The correlations between heterozygosity and growth related traits were investigated within each cross. A few significant relationships were observed. Namely, in the "Arcachon" cross a negative correlation was observed between heterozygosity and total weight. Further analyses are in progress.

2.1.5. Microsatellite marker analysis (P5)

The 68 oysters were sampled for microsatellite analysis on 3 loci. Genotyping is in progress.

2.2. Chromosomal studies

2.2.1. Aneuploidy and banding techniques (P3)

To assess transmission of aneuploidy from one generation to the next one, crosses were performed between oysters presenting contrasting levels of aneuploidy. As non destructive methods are not available, the parents were chosen from populations known to be contrasting. 3 "2 x 2" crosses were performed (P1a, P3) and the progenies are growing in La Tremblade. Aneuploidy level of these parents was very difficult to assess due to the low level of mitosis in the sampled tissues. Work is in progress.

Additionally, 6 full-sib families presenting contrasting growth, were sampled to study the relationship between aneuploidy and growth between families. To avoid bias, sampling was performed in two different ways: the mean weight within each family and the mean weight between the 6 families.

This year the G and R banding study was successfully completed meaning that we are now able to identify each of the different chromosomes of the *C. gigas* karyotype.

2.2.2. In situ hybridisation techniques (P4)

Following the development of a DNA genomic library, 28 probes were tested for *in situ* hybridisation on metaphase cells. Out of these 28 probes, 1 gave good results (i.e. single locus hybridisation, allowing the distinction of one chromosome pair) and 4 others could produce results with improved protocols.
The use of a cDNA probe of the amylase gene, developed by Partner 1d, was discussed.

2.3. Genetic study at the larval stage (P1a, P6)

A "5 x 5" cross was performed in order to test for gametic and zygotic competition at larval and spat stages. Parents were chosen from a population that had initially been genotyped using microsatellite markers, in such a way that the parentage could be determined in the progeny using only one locus. The relative contribution of each parent to the progeny was estimated at day 6, 18 and 90 after fertilisation. The results showed that gametic competition occurred between males ("spermatic competition") but not between females. Significant changes over time of relative parental contributions were recorded for both males and females. Further analyses are in progress.

2.4. Selection for physiological performances

2.4.1. Temporal stability of physiological performances (P1b)

28 oysters were studied for the temporal stability of their physiological performances (filtration and oxygen consumption rates) using a continuous monitoring system over 24h. These parameters were recorded on 5 dates between September 1997 and April 1998. A Friedman test demonstrates that the rank of the animals, is not significantly different over time for any of the parameters studied. The stability is due to a higher proportion of "stable" oysters than "unstable"ones.

2.4.2. Selection of the G1 parents for their physiological performances (P1b)

27 G1 oysters (one oyster died in May 1998) were ranked based on the mean of the 5 successive non-destructive results. The chosen parameter was the ratio of food absorption to oxygen consumption rates (expressed in terms of energy) rather than scope for growth. Therefore, the best oysters were those which assimilated the most energy and consumed the least.

2.5. production of the G2 generation families

2.5.1. G2 for aneuploidy transmission (P1a, P3)

Three "2 x 2" crosses, between oysters from populations presenting contrasting levels of aneuploidy, were performed in 1998 (P1a, P3). Parents were sampled to estimate their individual aneuploidy rates. The 12 full-sib progenies (≈ 3000 oysters/family except one family with less offspring) are growing in La Tremblade.

2.5.2. G2 for physiological performances (P1a)

The ranking of the G1 oysters studied for their physiological performances, based on the 5 successive measures, was available in June. Despite the fact that some of these oysters spawned spontaneously in their tank the 14th of May, 17 (3 females and 14 males) out of the 27 oysters spawned following a thermal induction on the 23rd of June. 20 full sib families were finally made by stripping of 2 contrasting females (number 1 and 26). Stripping was necessary in order to obtain enough female gametes. Out of these 20 crosses, 11 full sib families, called G2.1 were settled on cultch and are growing in la Tremblade.

The 26th of August, a second attempt was performed on the 19 remaining oysters. Seven male oysters spawned but no females. These 7 males were crossed with a single common female (which had not been studied for physiology). 7 full sib families, called G2.2 were settled on cultch and are growing in la Tremblade. The 19 oysters are still alive and a 3rd set of crosses is planned for February 1999. The following table summarises this information.

Oysters ranked June 9 (old number)	Rank (new number)	Spawners June 23	Spawners August 26	Status October 98
09B6	1	female		Stripped (June 98)
14B9	2	male	male	alive
13F3	3	male	male	alive
09C3	4			dead
15G4	5	male		alive
17C3	6	female		alive
14H3	7	male		alive
09C7	8	male		alive
13G9	9		male	alive
06B2	10	male	male	alive
13B8	11			alive
05H4	12			dead 19/08/98
17H5	13	male		alive
14F3	14			dead
13D7	15			alive
13F9	16			alive
13G1	17			dead
15A1	18	male	male	alive
17C7	19			alive
14F5	20			alive
09E7	21	male		dead 14/08/98
13B7	22	male		alive
14B7	23	male	male	alive
14H1	24	male	male	alive
06C9	25	male		dead
06B9	26	female		Stripped (June 23)
15G9	27	male		alive

3. Experimental plans agreed for 1999

Discussions were centred on the best use of animals available to all the partners.

3.1. Study of G2s for aneuploidy transmission (P1a, P3)

According to the results of the aneuploidy of the parents, one of the "2 x 2" crosses will be studied. Progenies will be sampled in two different ways: the mean weight within each family and the mean weight between the 4 families. Assuming 20 oysters per sample, this will be 20 x 4 x 2 = 160 oysters recorded.

Partner 2 will wish to study proteolitic activities on the same oysters. The question remains to be answered as to whether colchicine exposure biases the measurement of enzyme activities. A separate test should be performed beforehand to verify this point.

3.2. Study of G2s for physiological performances (P1b, P1d, P2)

4 full-sib families of the G2.2 will be studied: 2 "good" males and 2 "poor" males crossed with the same female. 100 progenies per family will be reared in Bouin in individual trays in order to record individual growth curves in a controlled environment up to April 1999, when oysters should weight at least 5 g. They will then be transferred to L'Houmeau or La Tremblade (according to the progress of the new lab being set up in L'Houmeau) and acclimated to low food availability over 1 month. Non-destructive study of physiology will be made on 30 oysters per family, then these will be dissected and sampled for proteolitic and digestive enzyme activity analyses.

4. Synthesis of the first 3 years

The results of the first three years were presented to the representative of DGXIV by the heads of each partner. This was found to be very useful for all partners as the project is spread over 5 years and is getting more and more complex.

5. General discussion

The following points were discussed:

- To set up a common database joining together all the data collected on the oysters from the « June 1997 experiment ». This database will be collated as an Excel file, by S. Bougrier and P. Boudry, for physiological and genetic data respectively and joined to the third year progress report.

- The relationships with American and Australian colleagues was discussed, in relation to the visit of Dr. Chris Langdon to IFREMER-Brest. The opportunity to organise a joint workshop on genetics and physiology of growth in *C. gigas* was discussed. This could take place after the next GENEPHYS meeting (1999) or at the last one (2000).

- It was proposed that the next GENEPHYS meeting could be held in Crete (if Partner 5 agrees !).

All participants expressed their thanks for the warm hospitality and very good organisation of the meeting by Noel Wilkins and Sophie Hubert.