Project n° 513692

Combined genetic and functional genomic approaches for stress and disease resistance marker assisted selection in fish and shellfish

AQUAFIRST

Instrument	STREP
Thematic Priority	1.3 Modernisation and sustainability of fisheries, including aquaculture-based production systems

Periodic Activity Report

Period covered:	2005-01-11 to 2006-31-10
Date of preparation:	2006-11-22
Contract Start Date :	2004-01-11
Duration:	42 months
Project coordinator name:	**Patrick Prunet**
Project coordinator organisation name:	**INRA Rennes**

DG Fisheries	**Project funded by the European Community**

Table of contents

Publishable executive summary

Type of contract: STREP		**Total project cost** (in euro): **5 821 320**
Contract number **513692**	**Duration** (in months) 42 Months	**EU contribution** (in euro) 3 799 954
Start date 01 November 2004		**Period covered by the project** 01 November 2004- 1 June 2008

PROJECT COORDINATOR

Name : **Dr Patrick PRUNET**	**Address :** **INRA/SCRIBE** **campus de Beaulieu** **35042 RENNES, cedex** **France**

Telephone : **(33)223-48-50-14**	**Telefax :** **(33)223-48- 50-20**	**E-mail address :** Patrick.Prunet@rennes.inra.fr

World wide web address: http://aquafirst.vitamib.com/

List of participants

CO01 (Coordinator) : Institut National de la Recherche Agronomique (INRA)
Responsible scientist : Patrick PRUNET
INRA/SCRIBE, campus de Beaulieu, 35042 RENNES Cedex, France.
Tel : (33)223-48-50-14
Mail : prunet@rennes.inra.fr

CO01.1: Institut National de la Recherche Agronomique (INRA)
Responsible scientist : Edwige QUILLET
INRA/Laboratoire de génétique des poissons, 78350 Jouy-en-Josas, France.
Tel : (33)1 34 65 23 93
Mail : edwige.quillet@jouy.inra.fr

CR02 : University of Aberdeen (UNIABN)
Responsible scientist : Chris SECOMBES
Department of Zoology, University of Aberdeen, 2 Tillydrone Avenue, AB24 2TZ Aberdeen, UK
Tel: (44)1224-272872
Mail: c.secombes@abdn.ac.uk

CR03 : The Natural Environment Research Council (NERC)
Responsible scientist: Tom POTTINGER
Centre for Ecology and Hydrobiology, Windermere Laboratory, Far Sawrey, LA22 0LP Ambleside, Cumbria, UK
Tel: (44)1524 595854
Mail: tgp@ceh.ac.uk

CR04 : Uppsala University
Responsible scientist: Svante WINBERG
Evolutionary Biology Centre (EBC), Department of Comparative Physiology, Uppsala University, Norbyvaegen 18 A, SE-752 36 Uppsala, Sweden.
Tel: (46)18-471-26-49

Mail: svante.winberg@ebc.uu.se

CR05: Ifremer La Tremblade
Responsible scientist : Sylvie LAPEGUE
Laboratoire de génétique et pathologie, Ronce les Bains, 17390 La Tremblade, FRANCE
Tel: (33)546762631
Mail: sylvie.lapegue@ifremer.fr

CR05.1: Ifremer Brest
Responsible scientist : Jeanne MOAL
Laboratoire de Departement de Physiologie fonctionnelle, Laboratoire de Physiologie des invertébrés, BP 70, 29280 Plouzané, France
Tel : (33)2 98 22 43 00
Mail: jmoal@ifremer.fr

CR06: CSIC
Responsible scientist : Jaume PEREZ SANCHEZ
Fish Growth Endocrinology, Instituto de Acuicultura de Torre de la Sal (CSIC), 12595, Ribera de Cabanes, Castellón, SPAIN
Tel : (34) 964319500
Mail: jperez@iats.csic.es

CR07: ICCM
Responsible scientist : Daniel MONTERO
Instituto Canario de Ciencias Marinas, Gobierno de Canarias, P.O. Box 56, 35200 Telde, Las Palmas, Canary Islands, SPAIN
Tel : (34) 928 132 900
Mail: dmontero@iccm.rcanaria.es

CR08: Wageningen
Responsible scientist : Hans KOMEN
Wageningen University, leerstoelgroep Fokkerij en Genetica, Bode 27, Postbus338, 6700 AH Wageningen, THE NETHERLANDS
Tel : (31) 317 4 82465
Mail: jmoal@ifremer.fr

CR09 : NUIG
Responsible scientist: Michael CAIRNS
Gene regulation group, National Diagnostic Centre, National University of Ireland, University road, Galway, IRELAND.
Tel: (353)91-512405
Mail: michael.cairns@nuigalway.ie

CR10: PML
Responsible scientist : Anthony J S HAWKINS
Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 3DH, UK
Tel : (44)1752633443
Mail: ajsh@pml.ac.uk

CR11: CCMAR
Responsible scientist :Adelino CANARIO
Comparative and Moleculare Endocrinology, Universidade do Algarve, Centro de Ciências do Mar, 8000-117, Faro, PORTUGAL
Tel : (351)289800925
Mail: acanario@ualg.pt

CR12: KULeuven
Responsible scientist : Filip VOLCKAERT
Katholieke Universiteit Leuven, Laboratory of Aquatic Ecology, Research Group on Fish Genetics, Ch. De Bériotstraat 32, B-3000 Leuven, BELGIUM
Tel : (32)16 32 39 72
Mail: filip.volckaert@bio.kuleuven.ac.be

CR13: HCMR
Responsible scientist : Giorgios KOTOULAS
Hellenic Centre for Marine Research Thalassocosmos, Former US base at Gournes, Gournes Pediados, 71500 Heraklion, Crete, GREECE
Tel : (30) 2810 337852
Mail: kotoulas@imbc.gr

CR14: UNIPD
Responsible scientist : Luca BARGELLONI
Department of public health, Comparative Pathology and Veterinary Hygiene, University of Padova, Viale dell'Università 16, Agripolis, 35020 Legnaro (PD), ITALY
Tel : (39)-049-8272506
Mail: luca.bargelloni@unipd.it

CR15: ROSLIN
Responsible scientist : Chris HALEY
Roslin Institute , Genetics and Genomics, Roslin, Midlothian, EH25 9PS, UK
Tel : (44)1315274432
Mail: chris.haley@bbsrc.ac.uk

CR16: CNRS
Responsible scientist : Francis GALIBERT
Faculté de médecine, Université de Rennes1, UMR-CNRS Génétique et Dévelopement, 35000 Rennes, FRANCE
Tel : (33)2 23 23 44 86
Mail: francis.galibert@univ-rennes1.fr

CR17: Nireus
Responsible scientist : Costas BATARGIAS
Molecular Population & Quantitative Genetics, Koumanioti 17, 26222 Patras, GREECE
Tel : (30) 2610 334626
Mail: batc@imbc.gr

Summary

The overall aims of the project is to identify, in sea bream, sea bass, oyster, and rainbow trout, genes of which expression is associated with disease and stress resistance and, from this information, to develop genetic approaches that allow characterisation of genetic markers for marker-assisted selective breeding of disease and/or stress resistant individuals.

For such project, the following main objectives will have to be reached:

- Characterisation in sea bream, sea bass, trout and oyster, stress- and disease-responsive genes as potential candidate gene markers for desirable traits;
- Seeking associations between (i) variations in response to stress and resistance to pathogen and (ii) selected candidates genes and microsatellites markers by segregation analysis in appropriate families (QTL analysis);

- Mapping of these genes in linkage and gene maps.

In order to characterize disease and stress-responsive genes in seabream, sea bass, trout and oyster, a functional genomic using microarray technology have been developed. During the second year of the project, all planned cDNA collections have been obtained and sequenced which allow the project, y combining his genomic resources with that of Marine Genomic Europe NoE to establish a database containing a large collections of EST for seabass, seabream, oyster and rainbow trout. These cDNA will be spotted on microarrays for seabream, seabass and oyster. For trout, this collection allowed the design of synthetic oligonucleotides which have been spotted in microarrays. All partners have been trained to analysis of microarray data and in vivo stress/pathogen exposure experiments have been carried out and validated. Gene profile analysis will be performed during the first part of the 3rd year.

During the second year of the project, we have also carried on several tasks devoted to characterization of genetic markers associated with stress or disease resistance. This includes search for SNP in stress or disease-resistant genes, production of relevant biological material (oyster and trout) on which a QTL analysis would be developed, development of new genetic markers (microsatellites) which would be later used for genotyping individuals in QTL protocols, construction of a radiation hybrid panel for sea bass.

Section 1 – Project objectives and major achievements during the reporting period.

General project objectives

Under intensive aquaculture conditions, fish are exposed to various stressors which are unavoidable components of the fish aquaculture environment. These stressful conditions (confinement, pathogen exposure, temperature...) lead to an overall reduction in performance, including poor acclimatizing and growth performance, impaired reproduction and increased susceptibility to disease. Moreover, to address these problems, growers often resort to an increased use of antibiotics and drugs. In shellfishery, significant mortality have been reported to occur during the summer months among both juveniles and adult Pacific oyster in several countries (Cheney et al., 2000) and are a major concern of oyster farmers (Goulletquer et al., 1998). Although stressful conditions have also been linked to mortality among oysters, the causes underlying this phenomenon are complex and physiological, environmental and pathological causes have been suggested.

Modification of the stress and disease responses by selective breeding has long been considered as feasible in vertebrates of economic significance. For oyster, which presents a large genetic diversity, selective breeding has led to the production of lines that are divergent for high or low survival during the summer period. With respect to aquacultured fish, several studies published in recent years clearly indicated the feasibility of increasing the tolerance of fish to stress by selective breeding. This may lead to a reduction in the adverse effects of stress, i.e. improved efficiency of the food conversion or reduced incidence of disease. However, while a recent EU-funded project has clearly demonstrated that it is possible to manipulate stress responsiveness in rainbow trout it also highlighted the practical limits to developing such a familial selection protocol in the long term. Similarly, in oysters, although the multidisciplinary program "Morest" (2001-2005), established in France to understand the causes of the summer mortality in *Crassostrea gigas* juveniles, allowed the observation of a very high heritability of survival, but still, long term selection protocols appear difficult to establish for such destructive character.

The overall aim of the project is to identify, in sea bream, sea bass, oyster, and rainbow trout, genes of which expression is associated with disease and stress resistance and, from this information, to develop genetic approaches that allow characterisation of genetic markers for marker-assisted selective breeding of disease and/or stress resistant individuals.

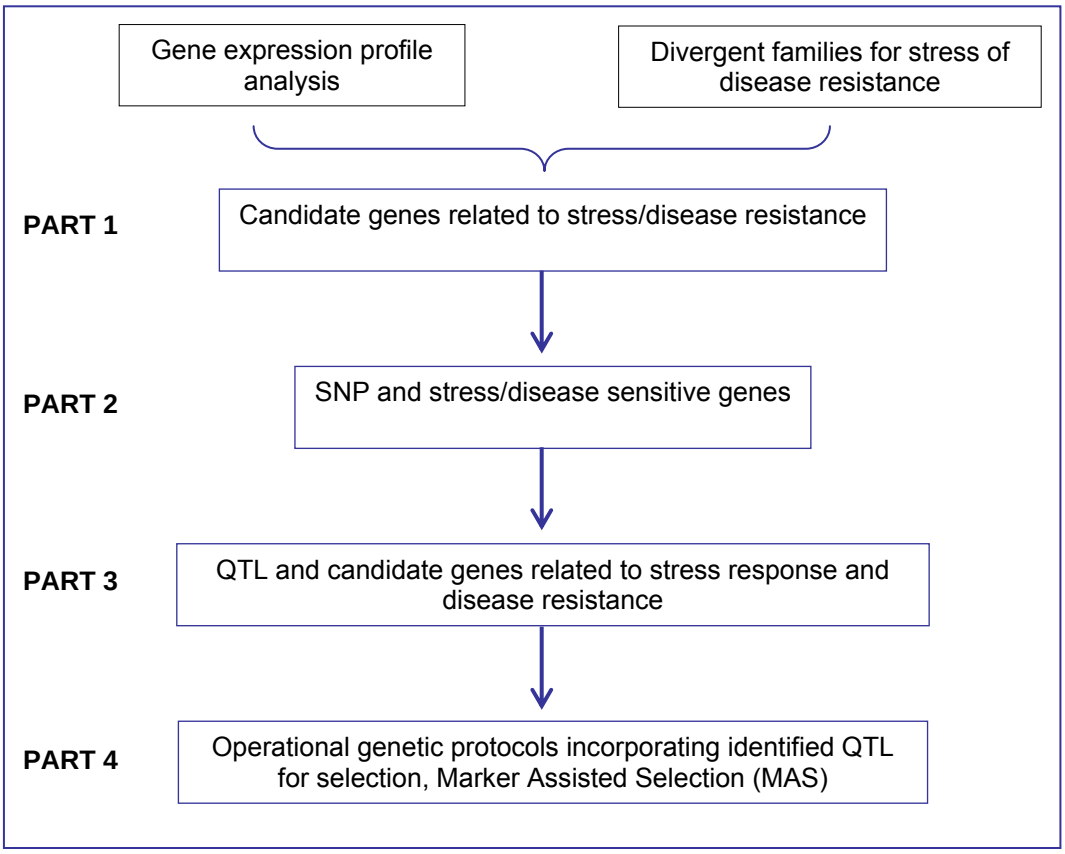
The originality of this approach relies on the successful association of
(i) functional genomic approach, i.e. development of microarray technology, to characterise disease and stress-responsive genes in the four species, and
(ii) evaluation of quantitative trait loci which have an effect on disease and stress resistance.
This requires mobilisation of a multidisciplinary research team gathering geneticists, physiologists and immunologists. The scientific strategy to be employed in this project will involve four complementary parts which will be developed over three and a half years:

Part 1: Identification and characterisation of genes involved in functional responses to stress or pathogen exposure.

Part 2: Characterisation of Single Nucleotide Polymorphism (SNP) in the candidate genes previously identified.

Part 3: Using a QTL analysis, identification of association between stress or disease resistance traits and these candidate genes and also linked microsatellites markers.

Part 4: Outline operational genetic protocols incorporating identified QTL and traditional breeding approaches in oyster, sea bream, sea bass and trout.



This strategy, which aims to associate functional genomic data and QTL analysis in order to characterise genetic markers, will be completely developed in trout and oyster, two species for which selection breeding has already been carried out and for which phenotypic divergent families for stress or pathogen resistance are available. In sea bream and sea bass, a similar strategy will be carried out and QTL analysis will use fish under selective breeding for growth. Moreover, the project will take advantage of the tools developed in flagship projects funded by EC "BRIDGEMAP" and "BASSMAP" addressing sea bass and sea bream. Based on low to medium density linkage and gene maps already obtained or under construction in these engaged projects, we plan to increase the density of sea bream and sea bass genetic maps. EST characterized by functional genomic analysis as being involved in stress or disease responses will also go onto the linkage and gene maps. Analysis of conserved synteny (a feature normally associated with conservation of function) together with functional genomic results can shed new light into candidate genes. With this approach and radiation hybrid technology in these species we expect at the end of the project to have the most dense gene maps of any aquaculture species and exceptional tools for QTL approaches and marker assisted selection (MAS).

These studies will be carried out on stress/pathogen situations related to aquaculture problems. Thus, in fish, we propose to work on acute confinement stress or exposure to pathogens specific of each fish species. In oyster, a major problem encountered in production plans is summer mortality which is a complex phenomenon related to possible environmental, physiological (gonad maturation) or pathological causes. For these stress

situations, several biological responses (traits) will be followed (for some of them, segregation between different families has already been observed and heritability measured). Finally, development of these physiological and genetic studies on similar stress situations in four marine species will favour the possibility of a comparative approach.

Operational genetic approaches to the utilisation of QTL and candidate genes in the breeding programme require to be specifically tailored for the species and its breeding programme and to the trait in question and its genetic architecture. For QTL conferring resistance to infectious disease, the epidemiology and population dynamics of the disease in question also need to be considered. Taking into account these factors and specific results and QTL identified in this project, we will develop example route-maps for the operational genetic exploitation of QTL in species studied which optimise the combined genetic progress for the traits of interest to the breeder.

Summary of the work performed during the second year

Part 1: Identification and characterization of genes involved in the functional response to stress or to pathogen exposure in oyster, trout, seabream and sea bass.

In order to characterize disease and stress-responsive genes in sea bream, sea bass, trout and oyster, a comon strategy using microarray technology has been developped. For these 4 species, the same strategy has been engaged: I) construction of relevant EST collections using SSH cDNA libraries ii) these EST or, for trout, synthetic oligonucleotide corresponding to EST sequences are further spotted on glass slides iii) finally, using these microarrays, gene expression profiles are analyzed in variuos tissues of animal exposed or not to stress or to pathogen. This approach has been successfully applied to trout during an on-going EU funded project (Stressgenes).

Construction of relevant stress specific EST collections (WP1).

EST collections were constructed following two strategies:

- For seabream, sea bass and oyster, we have been developping stress and disease-selective cDNA libraries using a supressive subtraction hybridization protocol (SSH libraries). This led to obtention of enriched collection of differentially expressed cDNA. For each species, cDNA SSH libraries were constructed using two tissues and by subtracting control and stress samples. In some case, this subtraction was carried out at two time points, early and late.
- For trout, we have been using the EST collections developped during the Stressgenes project and which was also obtained from several SSH cDNA libraries following the same strategy. In order to enrich this trout EST collection, further SSH libraries have been constructed within Aquafirst project: This include SSH cDNA libraries constructed with leucocytes exposed or not to cytokines in vitro and SSH libraries constructed from tissues collected on VHS sensitive or resistant trout exposed or not to the virus.

During the first year of the project, all in vivo and in vitro experiments related to this task has been carried out and RNA from tissues collected on control or exposed animals have been prepared. However, for oyster, quality control of RNA using Agilent technology revealed unexpected degradation of the RNA. This appeared to be related to the use of Dnase treatment in the protocol used for preparing RNA. By mutual agreement between

partners, the quality of these RNA was considered to be insufficient for their use in the SSH protocol. Finally, during the second year, a serie of new extraction using remaining tissues collected in 2005 was carried out and allowed us to recover enough mRNA material for SSH protocols.

Construction of SSH libraries.

Following the initial plans, various cDNA libraries containing stress or disease-selective genes have been constructed in the 4 species. This lead us to obtain a rich EST collections in seabream, sea bass, trout and oyster. In trout, this EST collection was added to the one prepared during the Stressgene project. Thus, during the second year, cDNA libraries from the following tissues have been prepared:

- Trout: leucocytes (cells exposed to cytokines), spleen (fish exposed to VHSV).
- Oyster: digestive glands, mantle, gonads, hemocyte, muscle, gills (oyster exposed to summer mortality).
- Sea bass: head-kidney, brain (fish exposed to confinement stress), gill, brain (fish exposed to pathogens)
- Seabream: liver, head-kidney, brain, gill (fish exposed to confinement stress), head-kidney, intestin (fish expsoed to pathogens).

Sequencing of the EST collections and selection of EST for microarrays.

All the sequencing work has been carried out by the Max-planck Institute in Berlin. Sequences were further sent to the Aquafirst Bioinformatic Centre (SIGENAE team, INRA Toulouse, france) for quality control and selection of non-redundant clones and identification of contigs. Thus, this work allowed us to obtain the following results:

- In trout: 3298 new EST sequences.
- In oyster: 5533 new EST sequences.
- In sea bass: 6403 new EST sequences.
- In seabream: 8756 new sequences.

These sequences have been added to EST sequences previoulsy obtained in other EU-funded projects, Stressgenes or Marine genomic Europe NoE (MGE): Thus, in trout, the new sequences generated within Aquafirst (mainly from cDNA SSH libraries) were fused to the Stressgenes EST collections. In sea bass, seabream and oyster, the Aquafirst sequences were fused to EST sequences produced by MGE NoE.
Bioinformatic analysis of these sequences in the 4 species allowed SIGENAE team to identify a list of contigs which could be spotted on the microarray. Thus, for each species, we obtained:

- Trout: 3678 sequences representing the same number of contigs. From these sequences, design and synthesis of 3678 oligos has been carried out by a private company (Eurogentec).
- Sea bass: 17,600 EST sequences identified.
- Seabream: 19,000 EST sequences identified.
- Oysters: 9,200 EST sesquences identified.

For the 3 last species, most of the clones containing these EST have already been sent to the Max-Planck Institute in Berlin for sequencing: This will facilitate the production of PCR-amplified material for spoting by the same plate-form.

Conclusion.

This technical part, which is crucial for the development of the functional genomic studies planned in this project, has been completed at the end of the second year. Finally, we obtained very significant progress in the construction of new genomic tools for aquaculture species:

1) Large collection of EST for oyster, seabream and sea bass which has not been otbtained before by any group. This was made possible by an efficient collaboration between MGE NoE and Aquafirst. It is important to notice that our colleagues from MGE fairly accepted to delay some fo their work and wait for Aquafirst EST production this year. This collaborative work allowed production of unique genomic tools.
2) In trout, we have been producing a unique collection of oligos corresponding to stress and disease-related genes which is very complementary to the AGENAE trout microarray produced by INRA.
3) We have been developping an efficient functional genomic plate-form which rely on 2 major partners: The Max-Planck Institute in Berlin (for sequencing, clones management and amplification, spotting, database management) and SIGENAE team in Toulouse for all the bioinformatic work related to Aquafirst. It would be interesting to consider how this network could be maintained after the end of Aquafirst project. This question could be tackled within Aquagenome project of which one objective is the development of durable resources for fish and shellfish genomic.

The major problem faced during this first part was delay in producing various tools. Finally, we have about 1 year delay with respect to the initial plans. Several reasons can explain this delay, a too optimistic planning of the work to be done by partners, development of a coordination between aquafirst and MGE, some technical problems. However, the main question now is wether this delay swould have any impact of the rest of the tasks of the project. At the present time, we think that this delay will not have major impact on other deliverables of the project, although this will imply a very stricted timing in the development of the project.This will be explicited later in this report, in the following parts (manily WP3, WP4 and WP5) of the project.

Development of the microarray technology within the project.

Following the development of a Aquafirst Bioinformatic Resource Centre during the first year of the project, several important tasks have been carried out during the second year. This includes:

- Organization of a training cession open to Aquafirst partners and dedicated to the use of bioinformatic tools necessary for analyzing and interpreting microarray data. These tools are available on the Aquafirst website which has a private access for Aquafirst partners.
- Establishment of collaborative links between MGE NoE and Aquafirst. In this context, the Max-Planck Institute in Berlin agreed to manage all oyster, seabream and sea bass clones belonging to MGE and Aquafirst and to carry out production of cDNA microarrays for these 3 species. Thisq work will be charged to each user at a low cost which make this solution acceptable for aquafirst partners.
- Planification of microarray production: 1) For trout microarray, the list of trout contig have been given to a private company (Eurogentec) who designed and synthetized oligonucleotides which have been further spotted on glass slides (3 times 3600 oligos/slides). Due to inexpected delay in the delivery of the oligos by Eurogentec company, these slides have only been produced during the last term of 2006. 2) For oyster, sebream and sea bass, selection of the clones to be spotted on the array have been carried out during the last term of 2006. We have planned with Max-Planck Institute production of the arrays for these 3 species between February and June 2007, according to their capabilities.

In conclusion, the Aquafirst project is able to produce trout oligos microarrays (corresponding to 3600 different contigs) and will produce in 2007 cDNA microarrays

for oyster (9000 different contigs), seabream (19,000 different contigs) and sea bass (17,600 different contigs).

Gene expression profiles during responses to stress and pathogen exposure.

The major goal of this activity is to characterize stress and pathogen-responsive genes using analysis of their expression profiles. Those genes which will be differentially expressed will be also relevant for our genetic analysis. During the first year of the project, several in vivo experiments were carried out according to the initial plan: This included I) exposure of two divergent lines of trout (high responsive –HR- and lowe responsive –LR-) to confinement stress ii) exposure of resistant or sensitive lines of oysters to hypoxia stress.

During the second year, complementary experimente were performed:

- In oyster, a bacterial challenge was done on the 2 lines (R and S) and various parameters (mortality, heamolymph analysis) indicated that the animal gave divergent biological responses as anticipated.
- In sea bass, juvenile fish were exposed to confinement stress or to pathogen. Analysis of expression of several candidate genes is now in progress.
- In seabream, the same type of experiments were alos carried out and a candidate gene approach was also developped on several genes, using real-time PCR technology.
- In trout, the two lines selected for their responsiveness to confinement stress (high- and low-responsive) have been exposed to pathogen (these experiments have been carried out during November 2006).

During the second year, all the in vivo experiments have been carried out and gene expression analysis using Aquafirst microarrays could then start at the beginning of the 3rd year. At this stage, this WP is about 6 months late on the original planning. Too large delay in the production of these results would be a problem for the next WP, particularly for the charcaterisation of SNP in trout and oyster. To solve this question, a very strict schedule has been decided:

- In trout, analysis of gene expression profile in head-kidney (confinement stress), skin and spleen (VHSV pathogen exposure), head-kidney and spleen (*Yersinia ruckeri pathogen exposure)* are in progress and will be finished for mid-February.
- In oyster, we have planned to hybridize all microarrays as soon as delivered by the Max-Planck institute in Berlin, at the latest before June 2007.

By following strictly this planning, the initial delay in production of microarrays should not have any negative consequences on the genetic part of the project.

Part 2: Characterization of SNP in stress / disease-sensitive genes.

Single nucleotide polymorphisms (SNPs) are the most common type of polymorphism in the human genome, with an approximate frequency of one every kilobase. These biallelic variants are relatively easy to genotype compared with VNTRs and microsatellites In marine bivalves, we expect to detect a high number of SNPs, considering that the allozyme diversity observed in these organisms is the highest recorded among eucaryotes generally. High genetic diversity has been also described in fish.

During the second year, partners involved in characterization fo this task have been continuing the work intiated during the first year:

- **In oyster,** the work carried out during the second year led to identification of 290 new SNP. Analysis of the polymorphism of these SNP in 24 F0 parents of families confirms the very high level of DNA polymorphism in this species, with an average density of 1.97-2.2 SNP/100 pb, a level among the highest ever reported to date. These SNP have been characterized from 56 EST sequences. The contribution of new EST corresponding to differentially expressed genes in sensitive and resistant

oyster families will be used during the 3rd year to increase this number of SNP. However, we plan to concentrate on these EST sequences and not to study promotor regions of these genes as initially planned. **Delay in delivering these differentially expressed genes (WP3) has no consequences on the characterization of SNP in oyster and their use in genotyping these markers in the F2 progenies (see part 3).**

- **In trout,** characterization of SNP has been started using EST sequences provided by partner 1 and obtained within the Stressgenes project (genes of which expression hae been modified after stress exposure). Due to the salmonid genome duplication, analysis of polymorphism by analyzing variability of the sequence chromatogram is not easy. To solve this problem, 36 selected genes have been sequenced on homozygous individuals provided by partner 1.1. Analysis of the polymorphism of these genes using DNA from the parents of the families used in the QTL study is now in progress. Thus, due to this complexity of SNP analysis in trout, it is important that we would focus on the most relevant genes related to stress resitance trait, i.e. those genes of which expression is differentially expressed in HR and LR trout lines. Similarly to oyster, we decided to concentrate on the coding region of the selected genes (EST) and not to analyze promotor regions of these genes, a task which will not be possible to carry out in time for SNP identification. In this context, we decided that the highest priority in the delivery of gene epression profile data by WP3 will be for trout. **The new planning proposed in the above paragraph will be in time to allow partner 1.1 to characterize new trout SNP as planned.**
- **In sea bass,** characterization of SNP have been initiated during the first year in the continuation of efforts initiated within the European Network of Excellence Marine Genomics Europe (see WP7 report). Development of new SNP for candidate genes in sea bass have been performed using in silico approach: 3 bioinformatic tools have been used and lead to identification of 85 SNP.

In conclusion, Identification of SNP in sea bass, oyster and trout is in progress and will be carried out in time so that these genetic markers could be used in the genetyping analysis in the QTL studies. Delay in delivery of WP3 information on gene expresssion profiles in trout and oyster will not have negative consequences on this task, as long as the project will be able to follow closely the new established planning. This will be the major objective for the coordinator during the 3rd year.

Part 3: Identification of QTLs and candidate genes related to stress and disease resistance in trout, seabream, sea bass and summer mortality in oyster.

QTL analysis is a powerful approach which will allow mapping loci that contains genes which affect quantitative traits. The strategy is to relate genetic markers (whatever their nature: neutral markers or known genes) to the phenotypic values of individuals in populations of adequate genetic structure. The 'marked' chromosomal segments are expected to contain genes with functional polymorphisms that affect the expression of the traits of interest. The access to biological material of known and wide phenotypic and genetic variability for the traits of interest is a key point to improve the power of QTL detection for a given experimental effort.

Production of biological material.

Production of biological material becessary to carry out this task in trout and oyster have been in started during the first year. For these 2 species, it was decided to produce F2 families issued from 2 F0 lines divergent for a spécific trait (responsiveness to confinement stress for trout, resistance to summer survival for oyster). Designs for these

QTL protocols have been optimized by patner 8 and 16 during the first year. Thus, during the second year, F2 families were produced for both oyster and trout.
Families required for QTL analysis for sea bass and sea bream were produced by a private hatchery (sea bass) and by partner 17 (Nireus).

Phenotyping and genotyping.

The first year of the project was mainly devoted to the optimization of the QTL design QTL analysis for each species. Based on the conclusions issued form this analysis, the practical design was set up during the first year and carryed on during the second year.

In trout, the final possible design, taking account various pratical problems, will rely on 5 families of 200 individuals which will be challenger for stress (confinement, salinity exposure, pathogene exposure) during the 3rd year.

In oyster, field and laoratory test have been performed on the F2 progeny. The analyzed challenger was response to summer period (dead or alive). As a result, summer mortality did occur in the laboratory conditions as expected and in relation to the presence of Herpes virus. However, in the field experiment, no mortality was recorded. Despite these results, it appeared that the statistical power of the design is enough to persue with the families which showed mortality in the laboratory experiment. Genotyping analysis of these families will be carried during the 3rd year.

For the QTL mapping of disease resistance in seabream, as decided at the end of the first year, a new experimental challenge was performed during the second year. Moreover, in order to have a precise view on the number of contributing parents to the experimental population issued from the use of a mass spawning protocole, a limited number of individual bave been genotyped for 6-9 microsatellite. This analysis showed that a sufficient number of fish in the broodstok were contributing to the experimental population. Genotyping analysis will be carried on during the 3rd year as initially planned.

For the QTL mapping of the stress response in sea bass and seabream, part of the challenge experiment has been carried out during the first year (sea bass) but for seabream it appeared necessary to repeat the stress experiment. This was done during the second year. Moreovern progress have been done on the phenotyping of the stress fish, i.e. on the measurement of plasma cortisol level. This task will be completed by early 2007. **The 6 month delay in delivery of these phenotyping data will not have any negative impact on the overall QTL analysis to be carried out in WP11.** For the genotyping analysis, choice of markers for whole genome QTL scan in sea bass and seabream is progressing and this work will be carried on during the 3rd year.

Improvement of genomic resources for seabream and sea bass.

This task is mainly covered by WP8, WP9 and WP10 and aims to bring mapped genetic markers and new genomic tools which will be useful for the localisation of QTL on the genetic map in seabream and sea bass. The second year has been mainly devoted to the continuation of the work initiated within Bassmap and Bridgemap EC projects, i.e. improvement of the construction of a high resolution map for sea bass and sea ream. Moreover, this task also benefited from strong collaboration with Marine Genomic Europe which has been developping large EST collections for those 2 fish species.

During the second year of the project, the most important results in this task was **the successful construction of a radiation hybrid panel for sea bass:** During the first year, partner 8 in charge of this task faced several difficulties. Hopefully, during the second year, these problems have been overcome mainly because of the use of a new source of fish and the use of spleen as donor tissue for fish cells. The last results obtained are very encouraging and partner 16 is now fully confident that they will be able to obtain 93 hybrid cell lines (sea bass/hamster) that should have a good retention value and provide a good representation of the sea bass genome. This tool will be used during the 3rd year for mapping a certain number of genetic markers.

QTL analysis and framework for QTL exploitation in seabass, seabream, trout and oyster.

During the first year, most of the activity of this part have been devoted to the finalization of experimental designs for QTL analysis in the 4 species. These results were presented at the Genetic in Aquaculture meeting last June 2006 in Montpellier (France). Along this line during the second year, partner 8 and 15 carry on this advising work in helping designing optimal protocols for oyster and seabream QTL analysis. In addition, partner 15 alos provide secure database for pedigree, marker and trait data for QTL analysis in oyster, seabass and seabream. For trout, we will be suing the MAPGENA database developped by INRA and provided through partner 1.1.

Conclusion.

Although delay in delivery of functional genomic data occurred for part 1, we think this should not have impact on the success of the genetic part of the project, i.e. identification of QTL related to stress and disease resistance in the four species. However, as this task has to be completed for April 2008, we will have to be very watchful and avoid any further delay in the development of this part.

Section 2 – Workpackage progress of the period

WP01 - Characterisation of stress or disease-responsive genes

Start date or starting event			1					
Activity type [1]			RTD/Innovation					
Participants	CO01	CO01.1	CR02	CR04	CR05.1	CR06	CR07	CR09
Person Months	16	1.5	9.6	15	17.1	8.26	8	24

Objectives

The main objective of this workpackage is to generate banks of EST clones from sea bream, sea bass, oyster and rainbow trout that are implicated in either confinement stress or pathogen exposure. To achieve this selection of clones a number of SSH libraries will be constructed from relevant tissues of each species. Clones will be selected for a final microarray (WP2) by screening and "cherry picking" of preliminary microarrays in order to reduce redundancy in the final array. In addition, for rainbow trout, the current Stressgenes project will provide a ready-made collection of relevant clones

Progress towards objectives and deviations from plan

Task T1.1: Preparation of RNA from stress/pathogen exposed animals

Task T1.1 had been completed in 2005 and reported in D1.1, 2005 except the oyster RNA preparation which is repoted here.

Oyster RNA preparation (partner 5.1)

1. Material and Methods

-Brief summary of RNA extraction problems encountered in 2005

In 2005, families R and S were sampled for SSH during a spring-summer in situ rearing and just before a summer mortality event (2 main dates in June). In 2005, there were problems to purify total RNA from these samples with the Qiagen- RNeasy Maxi Kit. Briefly, total RNA was extracted from six tissues by Partner 5.1 and sent to Partners 9 and 10 (November 2005). The RNA quantity was estimated by OD measurement. Absence of DNA carryover was checked by real time PCR (see report 2005). However, on receipt of the samples, Partners 9 and 10 checked the extracts using Agilent technology and detected a degradation of these RNA extracts. After several tests, the DNAse treatment was defined as the cause of this degradation. By mutual agreement between partners, the quality of samples was considered insufficient to start the SSH protocol and new RNA extracts were needed. After some trials, the possibility to extract the remaining tissue of same samples kept in Extract-all reagent at –80°C was considered.

-New RNA extraction

The remaining tissues used for the first extraction had been preserved in Extract-all (Eurobio) at –80°C and it was decided to re-extract the total RNA from these remaining tissue samples. Triplicates samples (similar to 2005 SSH samples) from the 2 dates before mortality were therefore re-extracted using Extract-all reagent. The protocol used for total RNA extraction was similar to that applied in the first extraction excepted that the extracting solution volume was increased up to 5 ml, to reach a concentration of 1 ml/50 mg of tissue (instead of 1 ml/150 mg of tissue). This was done to obtain a better RNA quality, drastically reduce DNA carryover and therefore avoid the DNAse treatment. RNA concentrations were measured at 260 nm using the conversion factor 1 OD = 40 µg/ml RNA, and RNA quality was checked by electrophoresis through a denatured agarose gel.

A real-time PCR assay was performed in duplicate with 0.5 µg total RNA in a total volume of 15 µL to test the presence of DNA carryover using *actin* and *elongation factor I* primers (Bacca et al., 2004; Fabioux et al., 2004). The concentrations of the reaction components were as follows: 0.33 µM each primer, 1.5 µL fluorescein and 1X Quantitect SYBR Green PCR kit (Qiagen). This reaction was performed using Taq Polymerase as follows: activation at 95°C for 15 min followed by 45 cycles of 30 sec at 95°C, 1 min at 60°C, and a melting curve program from 95°C to 70°C by decreasing the temperature 0.5°C every 10 seconds (protocol in Huvet et al., 2004). Each run included a positive cDNA control and blank controls (water) for each primer pair.

- mRNA enrichment

Each partner processed the RNA samples to constitute the pools R and S for each tissue (a mix of two replicates per date was used for SSH). mRNA was then purified from these pools of total RNA using the Quickprep micro mRNA purification kit (Amersham). Tests were previously done by Partner 5.1 to compare the mRNA purification with two kits: this Quickprep kit and the Oligotex mRNA kit (Qiagen). The efficiency on RNA extracted from oyster tissues appeared higher with the Quickprep micro mRNA purification kit (Amersham) (recovery: 1.9% versus 0.9%).

Despite the high number of animals which were used for the hemolymph sampling, the total RNA quantity obtained was too weak to be directly used in SSH and an additional enrichment step was added before doing the SSH experiment. A BD SMART PCR cDNA synthesis kit (Clontech) was used, which is based on PCR and allows the production of enough cDNA from a low quantity of starting material (less than 2µg of total RNA) to do SSH experiments. For each sample (R and S), 1µg of total RNA extracted from hemolymph was therefore used to synthesis cDNA following the BD SMART PCR cDNA synthesis kit procedure (Clontech).

2. Results and discussion

- Analysis of problems on RNA extracts November 2005

Control tests on the RNA samples extracted in 2005, showed that the degradation of these total RNA extracts was related to the DNAse treatment performed after extraction of total RNA. Further tests have shown that it was possible to extract RNA from remaining tissues which are the pellets of the first extraction. So, it was then decided to extract total RNA from the remaining tissues of the first extraction with a higher concentration of Extract-all reagent to decrease significantly the amount of DNA carryover and therefore prevent the need to perform a DNAse treatment.

- New RNA extraction

The 12 hemolymph samples from the first extraction (2005) were of good quality, especially because they were not DNAse treated and were not reextracted. Samples in triplicates from 25th of May (date 3) and 6th of June (date 4) which corresponded to dates near the mortality event were re-extracted for SSH for the other tissues (gills, muscle, digestive gland, gonad, mantle) (60 samples). From the re-extraction, the obtained quantities of total RNA were highly variable but considered sufficient for SSH and the quality appeared high on denatured agarose gels. The 2 replicates of the 2 dates were mixed equally to obtain a sample of "R" RNA and a sample of "S" RNA. A minimum of 200µg was obtained for each pool, except for muscle (around 140 µg).

After checking the total RNA quality by denaturing gel electrophoresis, these samples were deemed suitable to continue the SSH experiment. They were sent on dry ice to the Plymouth laboratory (P10) (gills and muscle) and to Galway University (P9) (mantle and digestive gland) on the 18th of January 2006. This result validates the choice to increase the volume of extracting solution to increase the quality of the extracted RNA. This protocol allowed avoidance of DNAse treatment which was suspected to have degraded the first RNA extracts. Indeed, from these pools of total RNA, real time PCR was performed on non treated total RNA using *actin* or *elongation factor I* primers. Results showed no amplification in each sample and for each primer pairs (whereas positive controls gave standard values of mRNA levels) which confirmed that no DNA carryover was present in them. RNA from the two pools of gonads (R and S) was kept in Brest for further SSH studies.

- Enrichment in mRNA

Polyadenylated RNA of gonad (P5.1) was isolated using a Quickprep micro mRNA purification kit (Amersham), measured at 260 nm and quality checked by Agilent. About 2% mRNA was obtained from total RNA.

For hemolymph, two pools (R and S corresponding to a mix of 4 replicates each, 2 per date (dates 3 and 4) of hemolymph samples have been processed using a BD SMART PCR cDNA synthesis kit (Clontech). Problems were encountered after PCR amplification and RSAI digestion during the purification step, with a lesser efficiency for S samples compared to R samples. The protocol had to be repeated 3 times to obtain for the S hemolymph sample and equilibrated with R sample to be processed for SSH.

Task T1.2: Construction of SSH libraries

Oyster

12 subtracted libraries were prepared by 3 Partners (P5.1, 9 and 10).

Partner 5.1 undertook the construction of 4 different subtractive libraries from two tissues: hemolymph and gonad. Subtractions were performed in both directions for comparison between R and S progenies. For this partner, 1,920 clones were sent to the MPI (Berlin) to be sequenced, corresponding to 672 clones from the gonad and 1248 clones from the hemolymph.

Partner 9 constructed 4 SSH libraries from digestive gland and mantle-edge tissue and as an initial screen for quality, 3,072 cDNA clones were picked, duplicate glycerol stocks made, PCR analysis carried out (on 192 cDNA clones) and all 3,072 clones sequenced.

Partner 10 constructed 4 SSH from muscle and gills and again 3,072 clones were sequenced.

Sequencing of oyster clones

After sequencing, a total of 5,533 valid sequences (of the 8064 sequenced clones) were obtained. Subsequent database searches using the BlastX and BlastN programs were performed, and contigs were built in collaboration by MPI and Sigenae (INRA, Toulouse) on Aquafirst ESTs, MGE ESTs and ESTs from public databases. In total, 1,758 contigs were obtained plus 7,518 singletons corresponding to a total of 9,216 ESTs available for slide spotting.

Trout

SSH libraries from VHSV exposure (Partner 1):

Two SSH (forward and reverse) were performed from spleens of VHSV exposed resistant/non exposed susceptible trout sampled at 3 days post-infection.

Total RNA isolation was performed by Partner CO1 from all the samples provided by Partner CO1.1 from the task 1.1. Then total RNA isolates of spleen sampled at day 3 post-infection were pooled in two groups: VSHV exposed R individuals and non exposed S individuals. As the amount of mRNA isolated was not sufficient to perform SSH with a standard protocol, an amplification of cDNA from spleen total RNA was performed using the Clontech's BD SMART PCR cDNA synthesis kit.

The amplified cDNA was then used as template for SSH library construction using Clontech's PCR Select Subtraction kit. Two SSH libraries were constructed by Partner 1 using this protocol.

The SSH quality was controlled at each step following the supplier's instructions and by using an mRNA control. SSH efficiency was checked comparing PCR amplifications of some control genes (housekeeping genes and genes known as differentially expressed between the 2 situations). The results observed on an agarose gel indicated that the libraries were of good quality. Indeed a reduction in the abundance of GAPDH cDNA was observed in the two libraries. Moreover the VIG-2 gene was used as a control of enrichment of differentially

expressed genes as it is known as being overexpressed in VHSV infected fish[1]. An increase in the abundance of VIG-2 cDNA was observed in the forward library (Rinfected/Snon-infected) and a reduction in the abundance of this cDNA was observed in the reverse library (Snon-infected/R infected) that seemed to confirm the efficiency of the SSH was rather good.

SSH libraries from cytokine stimulated trout head kidney leucocytes (Partner 2)
Three new recombinant trout cytokines (interferon-gamma, IL-1 beta and IL-15) were prepared to stimulate trout head kidney leucocytes. Four full-length SSH libraries from the cytokine treated samples were constructed and 1,920 clones sequenced from both directions. More than 1,000 unique contigs were produced, 700 of which were novel and added to the Aquafirst microarray selection (20% of the microarray).

Sea bream

Twenty SSH libraries were prepared, and 10,752 clones selected and sequenced, yielding 8,756 valid sequences.

Partner 9 made 8 SSH libraries from confinement stress and after initial analysis for quality, 4,608 cDNA clones were picked, duplicate glycerol stocks made, PCR analysis carried out (on 768 cDNA clones), and 4,224 clones sequenced.

Partner 9 also made 4 SSH libraries from pathogen exposure and after an initial screen for quality, 3,072 cDNA clones picked, duplicate glycerol stocks made, PCR analysis carried out (on 384 cDNA clones) and 3,072 clones sequenced.

Partner 4 prepared 4 brain SSH libraries and 4 gill SSH libraries from early confinement, with 3,072 clones sequenced and 2,205 valid sequences returned.

Sea bass

Twelve SSH libraries were planned for confinement stress and pathogen exposure experiments for sea bass. 12 SSH libraries (4 by Partner CO1 and 8 by Partner CR4) have been constructed. 8,448 clones have been sequenced, yielding 6,403 valid sequences.

SSH libraries from Pasterella piscicida exposure – gill (Partner 1)
Half of the gill samples from the pathogen exposure experiment were sent from Partner 7 to Partner 1 on dry ice: 3 samples from the 6 obtained for each condition. Two SSH (forward and reverse) libraries were prepared from the gill samples of *P.piscicida* infected/non-infected seabass sampled at 5 days post-infection, in order to select genes involved in the late response to this pathogen.
Total RNA isolation from the samples was performed by Partner 1 with the Chomczynski technic (Chomczynski, 1987). In total, RNA was extracted from 6 samples (3 gills from infected fish and 3 from non infected fish at 5 day post infection). The quality of these RNA extracts were analysed by agarose gel electrophoresis. A DNAse treatment was applied to the 2 pools of RNA (infected/non infected) and mRNA was isolated using a Qiagen Oligotex mRNAMidi kit. mRNAs were then analysed for quality by an Agilent 2100 BioAnalyser (Agilent) and the quality found to be insufficient for use for SSH.
An amplification of cDNA from total RNA was then performed using the Clontech BD Smart PCR cDNA synthesis kit.
The amplified cDNA was used as template for SSH library construction using a Clontech PCR Select Subtraction kit. Two SSH libraries were made by Partner 1 using this protocol.
The SSH quality was controlled at each step following the supplier's instructions. SSH efficiency was checked by comparing PCR amplifications of some control genes (housekeeping genes and genes known as differentially expressed between the 2 situations).

Abundance of GAPDH cDNA decreased in subtracted libraries compared to unsubtracted ones. Moreover, abundance of HSP70 cDNA (expected to be differentially expressed between infected and non infected fish) seemed to decrease in the forward SSH (infected/non infected) library compared to non subtracted cDNA, whereas an increase was observed in the reverse SSH (non infected/infected) library. All these results suggested that SSH efficiency was rather good.

Cloning

The two libraries were then cloned using a TOPO TA cloning kit (Invitrogen) into the pCR2.1-TOPO vector. The ligation reactions were transformed into electrocompetent TOP10 *E. coli* cells. 576 colonies were selected for each library and incubated overnight in 150µL LB + Ampicillin medium. Results of PCR analysis on each clone, with M13 primers, showed that most of the clones contained an insert.

SSH libraries from confinement stress experiment –head kidney (Partner 1)

SSH libraries were constructed from the second confinement stress experiment performed by Partner 7, with 6 stressed fish, 6 fasted controls and 6 fed controls sampled at day 7. Three tissues were sampled: liver, gill and anterior kidney.

All these samples were sent from Partner 7 to Partner 1 on dry ice. Two SSH (forward and reverse) libraries were prepared from head kidney of stressed/fed control seabass sampled at day 7 in order to select genes involved in the late response to confinement stress.

Total RNA was isolated from the samples by Partner 1 with the Chomczynski technic (Chomczynski, 1987). In total, RNA was extracted from 12 samples (6 head kidney from stressed fish and 6 from fed controls). The quality of these RNA extracts was analysed by agarose gel electrophoresis. A DNAse treatment was applied to the 2 pools of RNA (stressed/controls) and mRNA isolated using a Qiagen Oligotex mRNAMidi kit. mRNAs were then analysed for quality by an Agilent 2100 BioAnalyser (Agilent) and the quality was found to be insufficient for use for SSH.

An amplification of cDNA from total RNA was then performed using the Clontech BD Smart PCR cDNA synthesis kit.

The amplified cDNA was used as template for SSH library construction using Clontech's PCR Select Subtraction kit. Two SSH libraries were constructed by Partner 1 using this protocol.

The SSH quality was controlled at each step following the supplier's instructions. SSH efficiency was checked by comparing PCR amplifications of some control genes (housekeeping genes and genes known as differentially expressed between the 2 situations).

Amplifications performed with GAPDH primers were not successful but HSP70 cDNA seemed to be enriched in the forward SSH (stressed/controls) libraries compared to non subtracted cDNA, whereas it seemed to decrease in reverse SSH (controls/stressed). These results indicated that efficiency of the 2 SSH seemed rather good.

Partner 4 constructed 8 brain SSH libraries from confinement and pathogen exposure, with 6,144 clones sequenced and 5,274 valid sequences returned.

The following Table summarises the SSH libraries constructed and used for sequence analysis, sequence reactions performed and the valid sequences produced. In total, 45 SSH libraries have been made from 4 species and used for sequence analysis. 32,000 sequence reactions have been performed, and 24,000 valid sequences have been produced from this task.

Species	Partner	Experiment	library name	Nb of Aq. seq	Nb of va Aq. Se
Trout	Rennes	Pathogen spleen early (R/S inf by HSV) fw	rtrs05	840	356
		Pathogen spleen early (R/S inf by HSV) rev	rtrs06	354	168
	Aberdeen	Interferon gamma treatment	rtay11	1536	1059
		interleukin-1 beta treatment	rtay12	1536	1142
		IL-15 treatment	rtay13	768	573
	Total			**5034**	**3298**
Sea bream	Galway	confinement liver early fw	gsgl01	576	510
		confinement liver early rev	gsgl02	576	507
		confinement liver late fw	gsgl03	576	414
		confinement liver late rev	gsgl04	576	432
		confinement head kidney early fw	gsgy01	768	694
		confinement head kidney early rev	gsgy02	768	694
		confinement head kidney late fw	gsgy03	384	320
		confinement head kidney late rev	gsgy04	384	315
		pathogen head kidney late fw	gsgy07	768	612
		pathogen head kidney late rev	gsgy08	768	697
		pathogen intestine late fw	gsgi07	768	701
		pathogen intestine late rev	gsgi08	768	655
	Uppsala	confinement brain early fw	gsub01	768	370
		confinement brain early rev	gsub02	768	595
		confinement gill early fw	gsug01	768	542
		confinement gill early rev	gsug02	768	698
	Total			**10752**	**8756**
Seabass	Rennes	Confinement head kidney late fw	sbry03	576	260
		Confinement head kidney late rev	sbry04	576	315
		Pathogen gill late fw	sbrg07	576	302
		Pathogen gill late rev	sbrg08	576	252
	Uppsala	confinement brain early fw	sbub01	768	618
		confinement brain early rev	sbub02	768	658
		confinement brain late fw	sbub03	768	548
		confinement brain late rev	sbub04	768	683
		Pathogen brain early fw	sbub05	768	709
		Pathogen brain early rev	sbub06	768	697
		pathogen brain late fw	sbub07	768	700
		pathogen brain late rev	sbub08	768	661
	Total			**8448**	**6403**
Oyster	Galway	summer mortality digestive gland rev	oygd09	768	673
		summer mortality digestive gland fw	oygd10	768	689
		summer mortality mantle edge fw	oyge09	768	670
		summer mortality mantle edge rev	oyge10	768	673
	Ifremer	summer mortality gonad fw	oyio09	384	249
		summer mortality gonad rev	oyio10	384	310
		summer mortality hemocytes fw	oyih09	768	61
		summer mortality hemocytes rev	oyih10	384	64
	Plymouth	summer mortality muscle fw	oypm09	768	514
		summer mortality muscle rev	oypm10	768	603
		summer mortality gills fw	oypg09	768	512
		summer mortality gills rev	oypg10	768	515
	Total			**8064**	**5533**

Task T1.3: Selection of ESTs for the microarrays

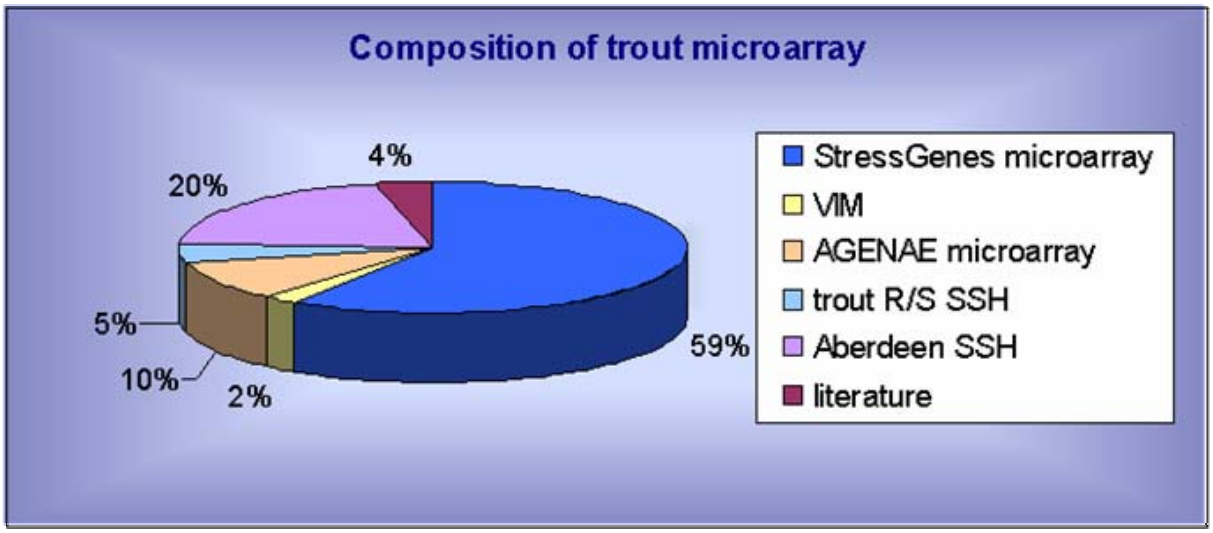
Selection of ESTs for the trout microarray

It has been decided to construct an oligonucleotide microarray for trout. Design and synthesis of the oligonucleotides, to spot onto the microarray, was made by Eurogentec from a list of sequences selected by Partner 1.
The sequences chosen to be spotted were selected from different sources:

- All contigs represented on the microarray constructed during the Stressgenes project were selected after elimination of the redundancy for the annotated ones.
- Sequences obtained from SSH libraries constructed by Partner 1 (pathogen exposure) were selected after elimination of the redundancy with the Stressgenes collection.
- The same work was done with the sequences selected from SSH libraries constructed by Partner 2.
- A hybridization was performed to select genes differentially expressed between stressed and control fish from the AGENAE generic macroarray.
- A list of VHSV-induced genes selected from infected/non-infected genes was given by the VIM INRA team (Jouy en Josas) and added to the selection.

- Interesting genes selected from literature.

Finally, 3,678 sequences were selected and sent to Eurogentec that managed to design a total of 3,342 oligonucleotides. 63% of these sequences have a homology with at least one protein.



Selection of ESTs for the seabream microarray

About 19,000 total contigs were obtained from the seabream assembly. One EST will be chosen in each contig to be spotted onto the microarray. The selection will be made by the Sigenae team and Partner 1. This work is in progress.

Selection of ESTs for the sea bass microarray

About 17,600 total contigs were obtained from the sea bass assembly. One EST will be chosen in each contig to be spotted onto the microarray. The selection will be made by the Sigenae team and Partner 1. This work is in progress.

Selection of ESTs for the oyster microarray

About 9,200 total contigs were obtained from the oyster assembly. One EST will be chosen in each contig to be spotted onto the microarray. The selection will be made by the Sigenae team and Partner 1. This work is in progress.

Progress towards objectives

Task 1.1 was finished in 2005 except for the oyster RNA preparation. The problem with oyster RNA preparation was resolved early in 2006 and RNA preparation completed early in 2006.

Task 1.2 was completed in 2006. 45 SSH libraries have been constructed and used for sequence analysis. 32,000 sequence reactions have been performed, and 24,000 valid sequences have been produced

Task 1.3 was also completed in 2006 and early in 2007. Selection of trout contigs to design oligos for the trout oligo microarray was completed in 2006. Selection of ESTs for other species will be completed at the beginning of January 2007.

Deviations from the project work program

There has been an overall delay of this workpackage because of the difficulty in obtaining materials in task 1.1. The whole workpackage was completed early in 2007.

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D01.01	RNA preparation from tissues from stress or pathogen exposed animals	3	January, 2006
D01.02	SSH construction	9	July, 2006
D01.03	EST selection and sequencing, PCR products generated	15	January, 2007

List of milestones

Code	title	Due date	Actual/foreseen achievement date
M1	For each species, banks of sequenced EST clones implicated in confinement stress or pathogen exposure.	12	2006

WP02: Development of a transcriptome plate-form

Start date or starting event		4			
Activity type [1]		RTD/Innovation			
Participants	CO01	CR02	CR04	CR05.1	CR09
Person Months	29.1	2.15	4	4	4

Objectives

The main objectives of this work package are (i) to accomplish construction of dedicated microarrays for each species (ii) to manage EST sequence data which have been generated by WP1 (iii) to manage microarray data produced by WP3.

Progress towards objectives

1- Development of a microarray management structure

During the first year of the project, we have developped for Aquafirst project a microarray management structure based on 3 components:

- A microarray plateform devoted to the construction of the microarrays for the projects.
- A bioinformatic resource centre which will be helped by INRA/SIGENAE team (Toulouse, France).
- A coordination of this structure which is managed by partner 1 (INRA Rennes).

The second year of the project was deveoted to the management of genomic data (see next paragraph). However, we have introduced several modifications in our functional plan of action:

- We have strengthen our collaboration with the Marine Genomic (MGE) NoE and particularly with his genomic resource center, the Max-Planck Institute –MPI- in Berlin (R. Reinhart): Initiated with the sequencing of EST produced by Aquafirst project, this collaboration lead us to also merge our EST sequence database for seabream, sea bass and oyster (trout was not involved in such process as MPI did not have any specific trout sequence information in their database). This merging leads to the construction of the largest oyster, seabream and sea bass databases presently available and which will be equally shared by the resource centers of the 2 European projects, MGE NoE and Aquafirst.
- Spotting of microarrays for Aquafirst partners have been slightly modified this year: Trout oligos microarrays have been spotted by the West Genopole microarray plate-form in Nantes or in Rennes, according to availability. This was managed so that spotting could not be a confounding factor when analyzing the trout microarray data. For seabream, sea bass and oyster cDNA microarray, MGI in Berlin appeared to be fully organized for carrying out this task: Both MGE NoE and aquafirst agreed to construct for each species a unique type of cDNA microarray and MPI in Berlin accepted to carry out this task. Thus, there will be a unique place for spotting oyster, seabream and sea bass microarray. For all species, we will be using glass slides.

The cost fo these spotting have been consequently updated but still appeared reasonnable: For trout, 4 partners (Galway, Aberdeen, Uppsala and Rennes) bought the oligos collection (about 30,000 Euros) and have also been charged 30 Euros/slide for oligos spotting. For seabream, sea bass and oyster, slide have been charged 120 Euros

which include cost of all steps fo the cDNA spotting technology, i.e. cherry-picking of the clones, PCR-amplification, filtration, spotting and quality control.

Planning of the microaray hybridization and scanning have been organized: Some partners are keen to carry out this task in their lab (Galway, Aberdeen, Uppsala, Rennes for trout or seabream), other are interested to benefit the INRA microarray plate-form in Rennes (IFREMER/Plymouth for oyster, Canarie island for sea bass).

2- Training course for microarray analysis.

The objective of this task is to develop training courses for microarray analysis. Partner1 (INRA/SCRIBE, Rennes) benefits from molecular and genomics facilities (IFR platform) and an INRA team dedicated to bioinformatics tools. This allowed him to develop in Rennes all experiences necessary for carrying out microarray analysis and to teach the interested partners the whole process, from microarray hybridization to analysis of the results.

One traning cession has been organized last November 2006 and has covered the following apsects:

- Education for the use of BASE, a comprehensive web-based database for the management of data generated by microarrays.
- Education for the use of TmeV, a verstatile microarray data analysis tool with algorithm for clustering, visualization, classification, statistical analysis and biological theme discovery.
- Training to analysis of sequences, batches and libraires (SURF tool).
- Training to aseembling processes and annotation (R-Icard tool).
- Training to contig analysis and manipulation using the Sigenae contig browser (Biomart and Ensembl tools).

This session was organized during 3 days and allowed participants not only to have formal presentation of the tools but also to practise using microarray data and aquafirst database. 13 participants coming from 9 different laboratories were attending this cession (IFREMER Brest and Montpelliers, PLM, Galway, CSIC, CCMAR. Concerning the traning for labelling of the probes, hybridization and scanning of the slides, this will be organized with the partners who will carry out their hybridization in Rennes just before the start of their experiments.

3- Production of glass slide microarrays

As indicated above, aquafrist partners involved in the functional genomic studies decided to merge their genomic resources with that of MGE NoE, at least for seabream, sea bass and oyster. Moreover, we also decided to construct all arrays related to these 3 species in the MGE plate-form. This decision and delay in the accomplishment of WP1 and production of EST collections (cf. previous WP report) induced a delay in the construction of microarrays for the project. However, advancement of this task for trout is much more advanced and now we have a rather good view of how these arraysw will be produced during the coming months:

- For trout: Based on the 3678 EST sequences previusoly identified (see WP1 report), we have ordered 3678 oligos designed by the manufacture (Eurogentec). This design was validated by the company using the trout Agenae database (containing 250,000 EST sequences corresponding to ~40,000 contigs) but several technical problems delaid delivery of the oligos collection. Finally, we received it in September 2006 which allowed us to rpoduce the first microarray slides for trout in October. According to the planning we have set up with the spotting platform in Rennes, all

trout glass slides requested for Aquafirst project will be produced by January 2007.

- For sea bream, sea bass nad oyster: These cDNA glass slides will be produced in Berlin (MPI). All EST sequences obtained from MGE NoE or from aquafirst have been merged by SIGENAE partner (Toulouse) and selection of clones to be spotted has been done. If we can recover all clones indicated in the Aquafirst database, we would be able to spot: ~9000 EST for oyster, 17,000 EST for seabream and seabass. We are now gathering these clones in Berlin for further spotting. This task will be carried out during the first semester of 2007.

Deviations from the project workprogram

Overall, this WP has about 1 year delay with respect to the initial plans. At the end of the second year, trout oligos microarrays are in production, although this production was delaid due to unexpected delay in the delivery of oligos by Eurogentec company. For the 3 other species, this is planned to be done by MPI in Berlin between February and june 2007.

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D02.01	Setting up the management of the microarray and bioinformatic plateforms	6	January 2005
D02.02	Training course for microarray analysis	9	December 2006
D02.03	Construction of microarrays for each species	12	July 2007

List of milestones

None this year.

WP03: Analysis of gene expression profiles

Start date or starting event				4					
Activity type [1]				RTD/Innovation					
Participants	CO01	CR02	CR03	CR04	CR05.1	CR06	CR07	CR09	CR10
Person Months	17	9.45	4.35	14	17	10.05	16	14	9.39

Objectives

The goal of this work package is to characterize stress and/or pathogen-responsive genes by analysis of gene expression profiles in challenged animals. Gene expression analysis is initially performed on RNA samples prepared from tissues obtained during wp1 (task 1.1). In addition, in rainbow trout and oysters where strains divergent in stress responses (trout HR and LR strains) and summer survival (oyster R and S strains) have been produced, gene expression profiling will be performed on individual animals from these divergent strains.

Progress towards objectives

HR and LR trout

F4 lines of HR and LR trout were reared from eggs provided by F3 parents in pooled gamete matings during January 2006. These fish were held at Windermere throughout 2006. In October 2006 25 individuals from each of the two lines of F4 fish were subjected to a 1 h period of confinement and then blood sampled (LR mean weight: 23.9 +/- 0.9g; HR: 16.8 +/- 0.7 g). Plasma cortisol levels were determined and it was confirmed that divergence in the plasma cortisol response to confinement was evident, consistent with the traits exhibited by the parent fish (post-stress plasma cortisol LR 10 ng/ml; HR 30 ng/ml).

In November 2006, 200 of each line (HR, LR) were transferred to Aberdeen for use in the planned disease challenge experiments.

Sea bass

Sea bass were subjected to stress and a pathogen challenge test and samples from kidney, spleen, gill, brain and liver tissues, were obtained. These samples, which are now are kept at -80ºC, will be used to identify candidate genes for stress and disease tolerance using the microarrays generated in the AQAFIRST project. Such studies on gene expression profiles are scheduled for spring 2007.

In addition, to genes to be identified in microarray studies, candidate genes have also been selected from databases.

Material and Methods:

Tissue samples of juvenile sea bass subjected to stress and pathogen challenge (Work package 1.1), were collected at different time points as detailed in Table 1. In Table 1, samples labeled 1-3 originate from three fish from this treatment and which were shipped to Partner 1 (Rennes, France) for EST experiments. Samples labeled 4-6 originate another three fish from the same treatment and these samples were kept in the lab of partner XX Canary Islands. A replicate of these samples has been used to check the viability (?) of some candidate genes.

Table.1. Number of samples per tissue and sampling

Experiment	Tissue	Sampling							
		0 hours	1 hours	4 hours	8 hours	1 day	2 days	5 days	10 days
Stress	Brain	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
	Liver	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
	Head kidney	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
Pathogen	Brain	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
	Spleen	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
	Liver	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6
	Gill	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6	1-3 4-6

Sampling procedure and storage of samples:

The samples were collected using autoclaved and DEPC (0.8%)-treated materials (scissor, microcentrifuge tubes, aluminum foil, etc.). Tissue samples were frozen in liquid nitrogen and stored at -80oC. RNA extractions. Samples labeled 4-6 were used for total RNA extractions. The RNA extraction was carried out by Quick PrepTM Total RNA Extraction Kit, following the supplier's instructions. RNA concentrations were estimated by absorbance at 260nm and degree of purity determined by the 260/280 nm ratio, which averaged 1.69. RNA quality was assessed on agarose gels (1%, TBE 1X) stained with ethidium bromide. Finally, the pellets were hydrated with 150 µl of Milli-Q sterile water, previously treated with DEPC at 0.1%, frozen with liquid nitrogen and kept at -80ºC.

cDNA synthesis:

cDNA was prepared using the primer pd (T)12-18 (Amersham Biosciences) and reverse transcriptase (RT) M-MuLV (Roche). All cDNA samples were reconstituted in a final volume of 50 µl containing dNTPs (0.5 mM), RT buffer (1X), pd (T) 12-18 (2mM), enzyme (0.5 U/µl) and 5 µg of total RNA. All reactions were performed at 37ºC overnight (8-10 hours) and the product was subsequently stored at -20ºC.

Candidate genes:

Several candidate genes were selected based on previously published results (Tab 2).

Table.2. Candidate genes

Gene	Reference
Heat shock protein 70 (HSP70)	Gornati et al. (2004)
Heat shock protein 90 (HSP90)	Gornati et al. (2004)
Inducible nitric oxid synthetase (INOS)	Wang et al. (2001)
11 β Hydroxylase (11 βH)	Hagen et al. (2006)
Glucocorticoid receptor (GR)	Terova et al. (2005)
Steroidogenic acute regulatory protein (StAR)	Frederick et al. (2004)
Cyclooxigenase-2 (COX)	Buonocore et al. (2004)
Growth hormone (GH)	Doliana et al. (1992)
Membrane-bound glycoprotein (CD8α)	Buonocore et al. (2006)

PCR conditions:

For all genes reported in Table 2, primer sets were designed using the Gene Runner software (v3.0) and PCR conditions were optimized according to magnesium chloride concentrations and hybridization temperatures (Tab 4). The primer sets amplified fragments that were between 100 – 200 bp long. Specificity was checked by sequencing all PCR products. The results from PCR experiments are summarized in Table 3.

Table.3. PCR of selected candidate genes

Gene	Primer sets	PCR conditions	Sequenced

	designed	established	products
HSP70	Yes	Yes	yes
HSP90	Yes	Yes	yes
INOS	Yes	No	no
11-βH	Yes	Yes	yes
GR	Yes	Yes	yes
StAR	Yes	No	no
COX	Yes	No	no
GH	Yes	No	no
CD8α	Yes	no	no
BA	Yes	yes	yes

Table 4. PCR conditions

Components	Candidate Genes				
	HSP70	HSP90	11-βH	GR	βA
Primer F	1 pmol/ µl				
Primer R	1 pmol/ µl				
Mg Cl2	2 mM	2 mM	1 mM	1 mM	1.5 mM
dNTPs	0.2mM				
Taq Pol.	0.04 units/ µl				
cDNA	3 µl				

Candidate Genes								
HSP70 / HSP90/ GR			11-βH			βA		
T	Time	Cycles	T	Time	Cycles	T	Time	Cycles
95ºC	5 s	1	95ºC	5 s	1	95ºC	5 s	1
95ºC	15 min	40	95ºC	15 min	40	95ºC	15 min	40
65ºC	30 min		65ºC	5 min		72ºC	10 min	
72ºC	10 min		72ºC	10 min		72ºC	30 min	
72ºC	30 min	1	72ºC	30 min	1	4ºC	∞	1
4ºC	∞		4ºC	∞				

Prior to analysis of gene expression by real-time PCR, the sequence of the amplified fragment was checked by ABI Prism® dRhodamine Terminator Cycle Secuencing Ready Reaction kit (Applied Biosystems). To that end, the PCR fragment was cleaned by Microspin™ S-400HR, following the instructions provided by the supplier (Amersham Pharmacia Biotech Inc.), and the sequencing reaction was carried out in a final volume of 5 µl (2 µl from kit, 5 pmol of the primer and 16 – 22 ng of the amplified product). The themocycler conditions for sequencing reactions are given in Table 5. Sequences obtained were checked against the database using Blast.

Table 5. The themocycler conditions for sequencing PCR fragments.

Temperature	Time	Cycles
94ºC	3 min	1
96ºC	10 s	30
50ºC	5 s	
60ºC	4 min	1
4ºC	∞	-

Oysters

In 2005, two experiments, focusing on two factors suspected to be implicated in summer mortalities i.e. hypoxia and bacterial infection, were realized and samplings performed for future hybridization on microarray. However, the results of the bacterial challenge experiment were unexpected in term of mortality and we did not get biological parameters confirming the success of this challenge. Therefore, a new pathogen exposure experiment was planned for 2006.

Summer mortality resistant (R) and susceptible (S) animals were produced in 2005. During the critical period (June 2006) when they were 15 month old, they were infected with a cocktail of live bacteria (V.aestuarianus, V.splendidus). As in the experimental protocol of 2005, sampling was performed at three times (12, 24, 48 hours post challenge), analysis were performed in quadruplicate and phenotypic parameters (bacterial concentration, hemocyte parameters as concentration of hemocyte populations and reactive oxygen species production) were measured to estimate the level of bacterial infection and by measuring hemocyte parameters revealing whether the oysters were in contact with bacteria.

Material and methods

Animals

Oysters were produced in March 2005 by divergent selection of inbred resistant and susceptible families. Pools of resistant (R) and susceptible (S) oysters were constituted and larval and post-larval rearings were performed at La Tremblade hatchery (Ifremer) till May. The stocks were then transferred to Bouin where they were allowed to grow in upwelling structures until the 28th of September 2005 (Degremont et al. 2005). The oysters were then deployed in the field in Normandy till the 20th of April 2006. This rearing protocol allows to avoid summer mortality during the first year and to maintain all phenotypes in resistant as well as in susceptible oysters. From April 20th to June 2nd 2006 they were conditioned in Bouin nursery in favorable nutritive and temperature conditions to allow a good gametogenetic development. At arrival on the June 2nd in Brest the shell was notched to facilitate the hemolymph bleeding. Oysters were placed in 500L tanks with circulating, temperature controlled seawater. The temperature was progressively increased from 17°C to 19°C and food was continuously distributed in tanks every day. On the 7th of June, 16 pools of 115 oysters of a total equivalent weight of 1800g for 8 pools S and 1900g for 8 pools R were constituted and distributed in 50L tanks filled with 20L filtered seawater (5µm), aerated by air bubbling. A mixture of live bacteria (V. splendidus and V. aesturianus) was then added at 5*108 bacteria/L. Algae (T-Isochrysis) were distributed daily in the tanks and the temperature was maintained at 19 ±1°C.

Bacteria

A mixture of two Vibrio strains (V. splendidus and V. aestuarianus) was used in the experiment. These strains were GFP transformed and chloramphenicol resistant (from Le

Roux F.) which permitted a rapid identification after spreading out on Marine Broth agar to control the quantity of bacteria in each tank after infection.

Bacterial challenge and samplings

The oysters, distributed according to resistant (R) or susceptible (S) to summer mortality, were either challenged by bacteria (RB, SB) or not (RT, ST) in 4 replicates /condition. In each tank, 25 oysters were reserved to follow the mortality.

Three samplings were performed at 12, 24 and 48h post challenge. For samplings, all material was DEPC treated and gloves were used to avoid RNAse contamination. The sets were treated sequentially: RB followed by RT then 2 hours later SB and ST to obtain the same duration of treatment for R and S.

For each sampling, hemolymph was withdrawn from muscle with a syringe and a 25 G gauge needle. After quality control under a microscope to eliminate dirty samples or those contaminated by germinal cells, the hemolymph was filtered on 80µm mesh and maintained on ice. A pool of 8 individual hemolymph samples was constituted by tank. An aliquot of 300µl was preserved for hemocyte parameter analysis by flow cytofluorimetry and another one of 20µl for bacteriological analysis. The rest of hemolymph was centrifuged at 2500 r/min during 15 min at 4°C to collect hemocytes. The supernatant was frozen at -80°C then stored at -20°C for further enzymatic analysis. The hemocytes were dispersed in 1.8ml cold Trizol and transferred in Eppendorf tubes and stored at –80°C for further RNA extraction.

Also, gills and mantle edge were dissected and a piece (around 30mg) was transferred to a tube containing 1.8ml Trizol. Tissue samples of 8 oysters were pooled for each tissue for one tank (around 240 mg of tissue). Tubes containing Trizol were mixed vigorously and allowed to stay in a freezer for 24h before freezing at -80°C for further RNA extraction.

The whole flesh of 2 animals by tank was placed in a tube with a cold Davidson solution for further in situ hybridisation analysis. After 24h at 4°C the Davidson solution was replaced by 70% ethanol and the tissue was embedded in paraffin.

Mortality was assessed once a day on the 25 oysters displayed in each tank to follow mortality until June 6th 2006. After the 48 hours challenge, the seawater was renewed twice a week and algae supplied daily. Test of mortality was performed by Chi square test on the total number of dead oysters per condition. Indeed for each condition, results were merged between replicates as chi square test did not reveal significant effect of replicate.

Measurements of immunological parameters by flow cytometry

Measurements of hemocyte types, numbers, and functions were performed on a FACScalibur flow cytometer (B-D Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Hemocyte parameter measurements (hemocyte mortality, total hemocyte and sub-population concentrations, phagocytosis assay, reactive oxygen species (ROS) production) were performed according to methods reported by Delaporte et al (2006). Results are expressed as number of cells per ml for total and differential hemocyte concentrations, and percentage of dead cells for haemocyte mortality. The phagocytic activity was expressed by the percentage of hemocytes that had engulfed three beads and more. Reactive oxygen species production was estimated by the mean fluorescence (in arbitrary units) detected in hemocyte sub-populations maintained in FSSW (basal ROS production). For each sampling time, statistical analysis (2-way ANOVA) were performed for each hemocyte parameter using STATGRAPHICS Plus 5.1 statistical software (Manugistics, Inc., Rockville, MD, USA), to test oyster phenotype and bacterial challenge effect. Percentage data were transformed (arcsin of the square root) before the 2 ways ANOVA, but are presented in tables as untransformed percentage values.

Results

Samplings

Samplings were performed as planned and tissues were directly extracted and stored at – 80°C in Trizol till further RNA extraction and hybridization on microarrays (table 1).

Table 1. Number of RNA samples per sampling time (T12, 24, 48 hours after the Vibrio challenge).

	T12	T24	T48
Hemolymph	16	16	16
Gills	16	16	16
Mantle	16	16	16
Digestive gland		16	
Muscle		16	

Mortality

Mortality was not related to the rearing conditions and there was no significant of bacterial challenge (Figure 1). Mortalities were observed rapidly in all experimental conditions after start of the experiment (7th June) and was around 5% at 48 h post challenge. No difference in mortality rate was observed between the control and the challenged oysters within either R or S groups. However, susceptible oysters (S) died significantly more than resistant (R) ones irrespective of conditions, challenged or non-challenged.

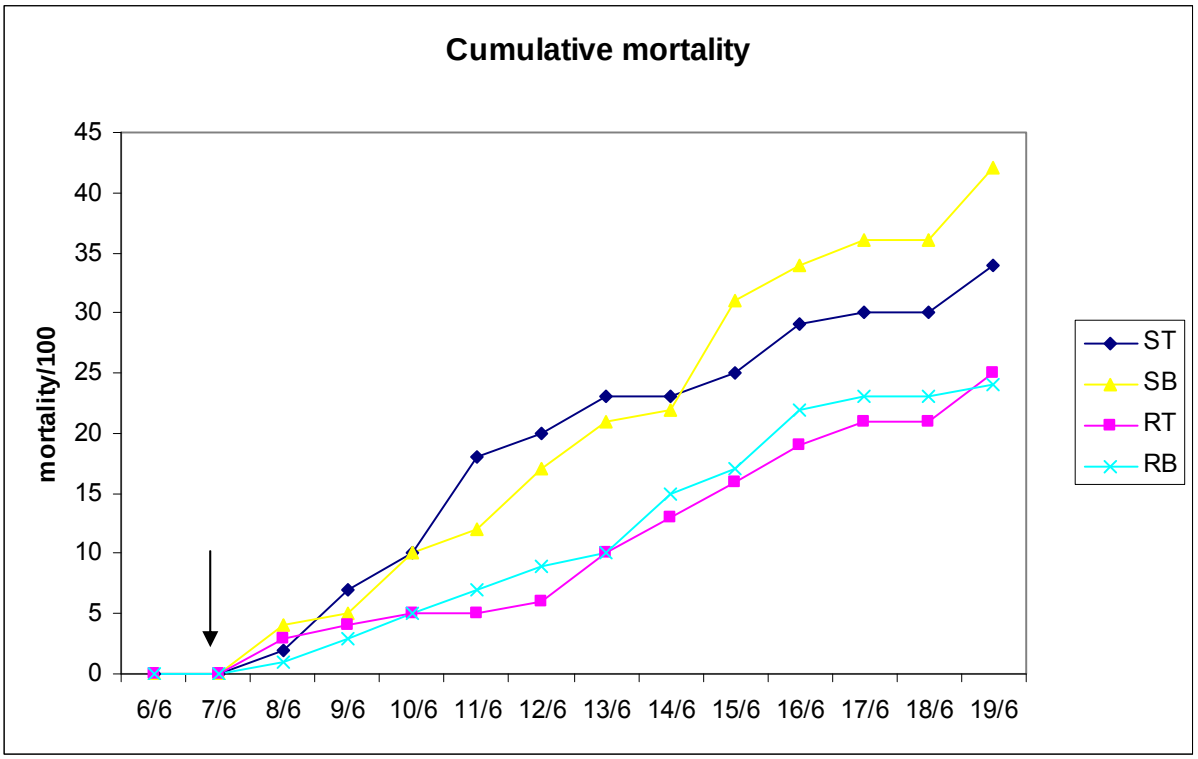


Figure 1. Cumulative mortality for R and S oysters after the bacterial challenge with live V.splendidus and V.aestuarianus (RB and SB) and for controls (RT and ST) not challenged by bacteria. The data represent the cumulative mortality in % per condition (4 tanks with 25 oysters each).

Bacteriology

The bacteria used for the challenge are GFP (green fluorescent protein) and resistant to chloramphenicol, allowing specific counts of bacteria in each tank in marine broth medium with antibiotics. An initial analysis of seawater immediately after addition of the bacterial mix showed that the bacterial population was constituted mainly by the strains GFP -V.aestuarianus and V.splendidus. After 48 H, the colony number of these strains in seawater was drastically reduced but the development of other strains was not observed.

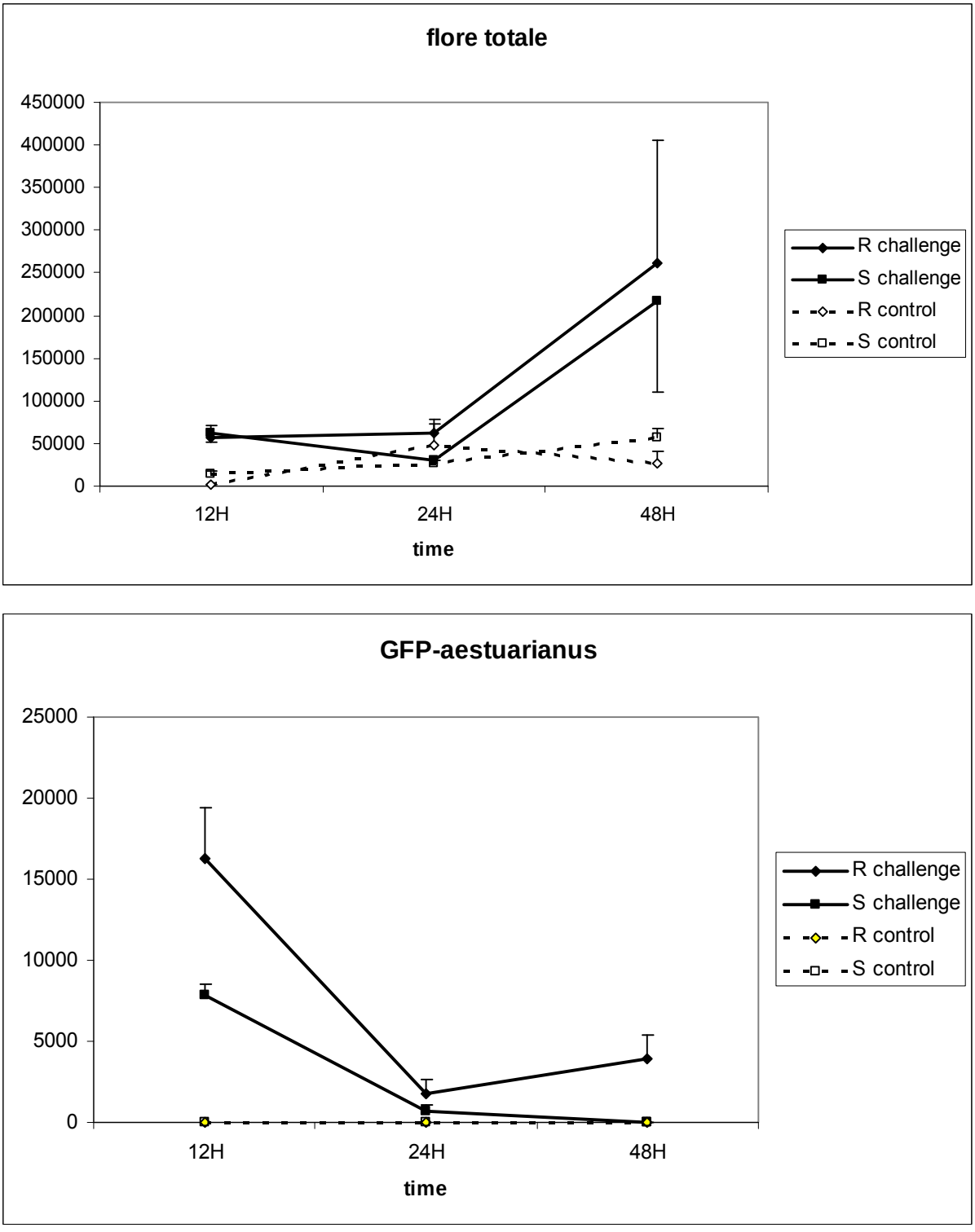


Figure 2. Estimation of bacterial concentration in oyster hemolymph . The data were obtained by counting colonies growing on marine broth agar without (Up) or with chloramphenicol, 25 µg/ml (Bottom).

In oyster hemolymph the GFP -*V.aestuarianus* and *V.splendidus* strains were rapidly detected (12 h after the beginning of challenge) with sometimes a wild strain of *V.aestuarianus*. However the strains used for the challenge disappeared from the hemolymph at 24 hours when wild strains of Vibrio increased sharply (Fig 2). Only data

on *V.aestuarianus* are presented because the exact enumeration of *V.splendidus* was not possible due to technical problems.

Hemocyte parameters

All parameters describing hemocytes were quantitatively different for R and S oysters. Whatever the sampling time and the condition (challenged or not), R oysters had significantly less circulating hemocytes, less phagocytic activity and less ROS production than S oysters (Table 2).

The influence of Vibrio on hemocyte parameters was more subtle and transient. For total hemocyte, granulocyte and hyalinocyte concentration, a very short impact was seen in challenged oysters exhibiting lower values than the unchallenged oysters after 12 h. Then, values of challenged and unchallenged oysters showed similar levels at 24 and 48 h. For hemocyte functional parameters (phagocytosis, ROS), the response appeared later but was also transient. After 24 h, these parameters were lower in challenged oysters compared to unchallenged ones but similar values are observed after 48 h.

Table 2. Summary of 2 ways ANOVA on hemocyte parameters measured by flow cytometry.

	12 H		24 H		48H	
	R/S	Bact/Control	R/S	Bact/Control	R/S	Bact/Control
[granulo]	R<S**	B<C**	R<S**	NS	R<S**	B<C
[hyalino]	R<S**	B<C**	NS	NS	R<S**	NS
[hemocytes]	R<S**	B<C*	NS	NS	R<S**	NS
mortality	R<S**	NS	R<S*	NS	R<S*	NS
phagocytosis	R<S**	NS	R<S**	B<C*	NS	NS
ROS (granulo)	R<S**	NS	R<S**	B<C*	R<S**	NS
ROS (hyalino)	R<S*	NS	R<S**	NS	R<S**	NS

*Analysis of variance for 2 factors, challenge and oyster phenotype at the different sampling times. ** probability 99%, * probability 95%, NS non significant. n=16 per test (4RT + 4RB/ 4ST+4SB) or (4RT+ 4ST/4RB+4SB). [granulo], [hyalino], [hemocytes] represent the concentration of granulocytes, hyalinocytes and total hemocytes and are expressed in cells/ml; hemocyte mortality and phagocytosis are expressed in percentage and ROS of granulocyte and ROS of hyalinocytes are arbitrary units.*

Discussion

A slight and continuous mortality was observed during the experiment whatever the oyster phenotype (R or S) and the experimental conditions, (challenged or not with potential pathogen bacteria). However, we noticed a difference in mortality rate between R and S families, a difference that is in accordance with their expected susceptibility to summer mortality. This "natural" mortality appeared independent of the bacterial challenge and could rather be explained by the physiological status of the oysters prior to the experiment. Indeed prior the start of the experiment, the oysters was reared in high trophic conditions to favor the gametogenetic development which is known to be highly involved in the summer mortality phenomenon. The macroscopic observation of oysters during dissection showed an advanced gametogenesis, confirmed by histological analysis (data not shown). Moreover the BMP15 gene analysed by quantitative PCR (Biorad) indicated a higher expression in S than R oysters which can be related to higher

gametogenetic development for S oysters than for R oysters. In fact in mammals this gene is implied in folliculogenesis. Our results are in accordance with previous results which indicate that the intensity of gametogenesis is positively correlated to increasing susceptibility to Vibrios.
It is unlikely that this regular mortality was associated to an infectious state prior the experiment as low concentration of Vibrio was detected in hemolymph at their arrival. The introduction of potential pathogen bacteria in the tanks did not induce an additional mortality during the first 48 h survey corresponding to the time of sampling for the transcriptome analysis. However the detection of exogenous GFP Vibrios in seawater during 24 h and their presence in oyster hemolymph suggests that a contact with oysters occurred. Moreover hemocyte parameters were transiently modified in oysters challenged with these Vibrios confirming the impact of the bacterial stress. The lower hemocyte concentration in challenged oysters compared to the unchallenged oysters may be related to an attraction of hemocytes towards local infection sites created by the vibrio challenge, lowering circulating hemocytes. Moreover the large differences in hemocyte characteristics between R and S oysters confirm the existence of physiological and immunological differences associated to summer mortality susceptibility.
Thus, the analysis of gene expression profiles using the functional genomic approach (microarray technology) in response to a bacterial challenge should help to identify genes associated with disease resistance in oyster and explain the phenotypic differences between R and S oysters.

Summary

In 2005, an experimental hypoxia was performed on R and S oysters. Two samplings were performed in tetraplicate at 2 and 10 days on 5 tissues (muscle, gonad, digestive gland, gill and mantle edge). A total 192 samples were obtained for RNA extraction and are stored in extract-all at -80°C. Also, an "in situ" survey in Auray (Brittanny, France) was performed on the same oyster families during the 2005 summer months. In this experiment samples from different tissues for RNA extraction were obtained at 5 different dates between May and July. In 2006, 176 samples were obtained from an experimental bacterial challenge performed on R and S oysters. Hybridizations of these samples on microarrays spotted in MPI (Berlin) are planned for 2007 on these samples. These experiments will focus on the gonad for the in situ experiment, on gills and muscle for the hypoxia exposure, and on gills and mantle for the bacterial challenge. In total 166 slides.

References

Dégremont, L., Bédier, E., Soletchnik, P., Ropert, M., Huvet, A., Moal, J., Samain, J.F. & Boudry, P., 2005. Relative importance of family, site, and field placement timing on survival, growth, and yield of hatchery-produced Pacific oyster spat (Crassostrea gigas). Aquaculture 249, 213-229.

Delaporte, M., Soudant, P., Lambert, C., Moal , J., Pouvreau, S. & Samain, J.-F. 2006. Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle. Aquaculture, 254 (1-4) 571-582.

De Zwaan A., P. Cortesi , G. van den Thillart, J. Roos, K.B. Storey, 1991, Differential sensitivies to hypoxia by two anoxia–tolerant marine molluscs: a biochemical analysis. Mar. Biol. 111, 34-351.

Gay, M. , Renault T., Pons A.M., Le Roux F.2004. Two Vibrio splendidus related strains collaborate to kill Crassostrea gigas :taxonomy and host alterations.Dis.aquat.Org.62 :65-74.).

Labreuche, Y., Soudant, P., Goncalves, M., Lambert, C., Nicolas, J.L., 2006. Effects of extracellular products from the pathogenic Vibrio aestuarianus strain 01/32 on

lethality and cellular immune responses of the oyster Crassostrea gigas. Dev. Comp. Immunol. 30, 367-379.

Tran D, A. Boudou, J.C. Massabuau, 2000. Mechanism for maintaining oxygen consumption under varying oxygenation levels in the freshwater clam Corbicula fluminea. Can. J. Zool., 78, 2027-2036.

Sea Bream

Objectives

Two experimental conditions were considered to analyze the gene expression profile in gilthead sea bream: confinement stress (i) and pathogen exposure (ii). For this purpose, the construction of a sea bream specific microarray was planned. The micrroarray was based on available sequences of public databases, EST collection of the Network of Excellence Marine Genomics, and stress and diseases libraries (16) constructed by suppressive subtractive hybridization (SSH) strategies (task 1.2 of WP01). During the second year of the project, selected SSH clones have been cloned and sequenced, and 19069 gilthead sea bream contigs are now available. Printing of microarray slides is in progress at the Max Planck Institute, and hybridization and gene expression analysis will be performed during the first semester of 2007 by partners CR06 and CR09 at the facilities of CR09 in Galway.

Progress towards objectives

Juvenile gilthead sea bream were exposed to confinement stress and to a myxozoan parasite (*Enteromyxum leei*) at the experimental facilities of partner CR06. Data on a range of physiological variables [respiratory burst activity and circulating levels cortisol, glucose, lactate, growth hormone and insulin-like growth factor) were reported in the first annual report (task 1.1). To better understand the intensity and quality of stressors, a candidate gene approach with selected target tissues (liver for confinement exposure, and intestine and head kidney for *E. leei* infection) was conducted during the second year of the project (Partner CR06). The gene expression profile was analyzed by real time PCR assays, and mRNA expression of specific genes were normalized against the expression of β-actin mRNA and related to the expression in control fish not subjected to stress. These target genes will also be included on the sea bream microarray that is now being developed.

RNA extraction and RT procedure

Total RNA extraction was performed with the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Tissues were homogenized in guanidine-detergent lysis reagent (25 mg tissue/ml). The reaction mixture was treated with protease K, and RNA purification was achieved by passing the tissue lysate (0.4-0.5 ml) through a purification tray containing an application-specific membrane. Wash solutions containing DNAse were applied, and total RNA was eluted into a 96-well PCR plate. The RNA yield was 40-50 µg for liver and 10-20 µg for intestine and head kidney with absorbance measures (A260/280) of 1.9-2.1.

Reverse transcription (RT) with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were reverse transcribed in a final volume of 100 µl. RT reactions were incubated for 10 min at 25 ºC and for 2 h at 37 ºC. Control reactions were run without reverse transcriptase and were used as negative real-time PCR controls.

Real- time PCR

Real-time PCR was performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as described in Calduch-Giner *et al.* (2003). RT reactions were conveniently diluted (1:7.5; 1:75; 1:375), and 7.5 µl were used for PCR reactions in a 25-µl volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad) with specific primers (Table 1) at a final concentration of 0.3-0.9 µM. For each experimental condition, 5-8 individuals were tested.

The efficiency of the PCR reaction for target and reference genes varied between 88 and 95%. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and fluorescence data acquired during the extension phase were normalized to cvalues found in control fish. Data on ß-actin (housekeeping gene) expression did not vary with stressor exposure.

Table 1. Forward (F) and reverse (R) primers for real-time PCR.

Gene	accession number		primer sequence	position
□-Actin	X89920	F	TCC TGC GGA ATC CAT GAG A	811-829
		R	GAC GTC GCA CTT CAT GAT GCT	861-841
GRP-75	DQ524993	F	TCC GGT GTG GAT CTG ACC AAA GAC	358-381
		R	TGT TTA GGC CCA GAA GCA TCC ATG	500-477
TNF □	AJ413189	F	CAG GCG TCG TTC AGA GTC TC	587-606
		R	CTG TGG CTG AGA GGT GTG TG	663-644
CYP 1A1	AF011223	F	GCA TCA ACG ACC GCT TCA ACG C	903-924
		R	CCT ACA ACC TTC TCA TCC GAC ATC TGG	1071-1045
IL 1□	AJ277166	F	GCG ACC TAC CTG CCA CCT ACA CC	499-521
		R	TCG TCC ACC GCC TCC AGA TGC	629-609
α2MG	AY358020	F	GCC AAA CTC GGT GCC TCT CCT ACT GC	676-701
		R	CTG CCC TGT GAG CCA TCT GAC AAT CGG	750-724
GR	AJ937873	F	TGT TCA GCC ACC CAC CCA TCG G	927-948
		R	GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG	1041-1012
GPx	DQ524992	F	GAA GGT GGA TGT GAA TGG AAA AGA TG	34-59
		R	CTG ACG GGA CTC CAA ATG ATG G	162-141
IGF-I	AY996779	F	TGT CTA GCG CTC TTT CCT TTC A	112-133
		R	AGA GGG TGT GGC TAC AGG AGA TAC	195-172
IGF-II	AY996778	F	TGG GAT CGT AGA GGA GTG TTG T	406-427
		R	CTG TAG AGA GGT GGC CGA CA	514-495
GHR-I	AF438176	F	ACC TGT CAG CCA CCA CAT GA	1275-1294
		R	TCG TGC AGA TCT GGG TCG TA	1373-1354
GHR-II	AY573601	F	GAG TGA ACC CGG CCT GAC AG	1690-1709
		R	GCG GTG GTA TCT GAT TCA TGG T	1764-1743

Confinement stress

GRP-75, also known as mortalin, mthsp70 and PBP74, is a member of the heat shock protein 70 family. In mammals, mortalin exerts relevant functions in cell survival, control of cell proliferation and stress responsiveness (*Kau et al.*, 2002). No data exists in fish on GRP-75 expression, and interestingly in the present study we found that hepatic expression of GRP-75 was rapidly up-regulated after confinement exposure (6, 24 and 72 hours) with a recovery to control values following 5 days of recovery (Fig. 1A).

Hepatic expression of pro-inflammatory cytokines (tumor necrosis factor α, TNF) and the oxidant agent cytochrome P450 1A1 (CYP 1A1) were affected by confinement exposure (Fig. 1B, 1C). The time course for the effect on these two genes was similar and opposite to that reported for GRP-74. Thus, the hepatic expression of TNF and CYP 1A1 remained unaltered 6 hours after confinement exposure, decreasing thereafter at 24, 72 and 120

hours. These results reflect a counterbalanced production and scavenging of reactive oxygen species (ROS) in order to preserve the redox-homeostasis in an oxidative scenario (confinement stress).

The modulation of ROS production and antioxidant defenses by the endocrine system has also been shown in different experimental models. Thus, in long-live dwarf mice, the reduced signaling of GH and IGF-I contribute to maintain and appropriate cellular redox state (Coschigano *et al.*, 2003; Holzenberger *et al.*, 2003). Conversely, fast growing animals over-expressing GH live shorter and combat oxidative stress less efficiently than normal and dwarf mice (Rakoczy, 2003). Experimental evidence also supports the involvement of the GH/IGF axis on the redox homeostasis in fish. Endocrine IGF is primarily of hepatic origin, and we found herein that confinement exposure reduced IGF-I and IGF-II expression at 24 hours of confinement stress with some recovery occurring at 72 and 120 hours following confinement (Fig. 2A, 2B).

Hepatic expression of GH receptors (GHR) was also analyzed, and we found a different gene expression pattern of GHR-I (Fig. 3A) and II (Fig. 3B). For GHR-I no effects on the expression were found over the sampling period, whereas the expression of GHR-II was down-regulated in stressed fish following the same time course as changes in hepatic IGF expression. This finding confirms the suggestion that two GHRs with a different expression pattern coexist in the same fish species. This assumption was further supported by sequence analysis of regulatory elements in the promoter region of GHR-I and II of gilthead sea bream (unpublished results).

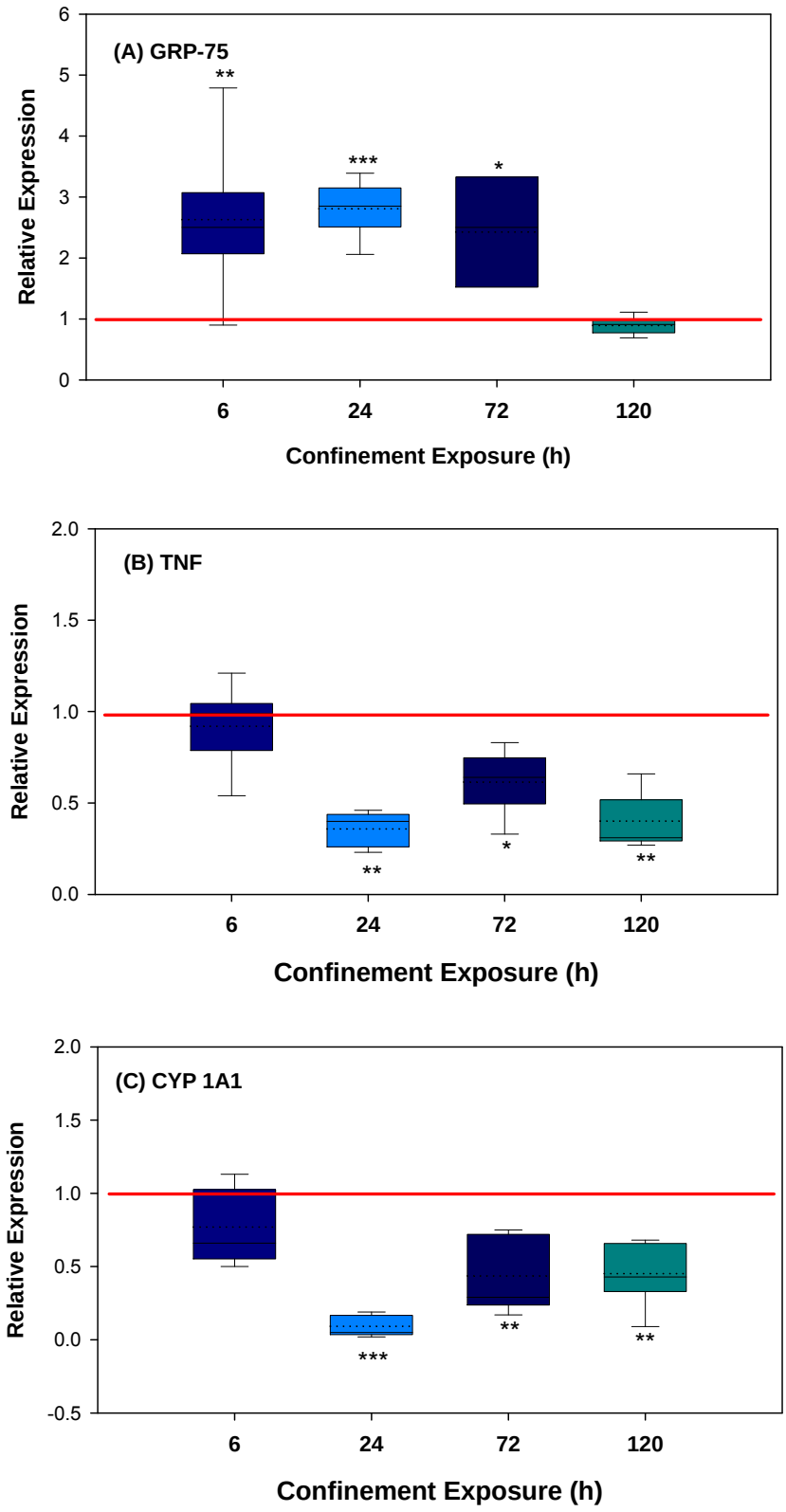


Figure 1. *Box-Whisker plot representing the relative gene expression of hepatic GRP-75 (A), TNF (B) and CYP 1A1 (C) after confinement exposure. Inside box, the dotted line represents the mean value; continuous line is the median. At each sampling time, data were referred to values of non stressed fish (Student t-test; *, >0.05: **, >0.01; ***, >0.001)*

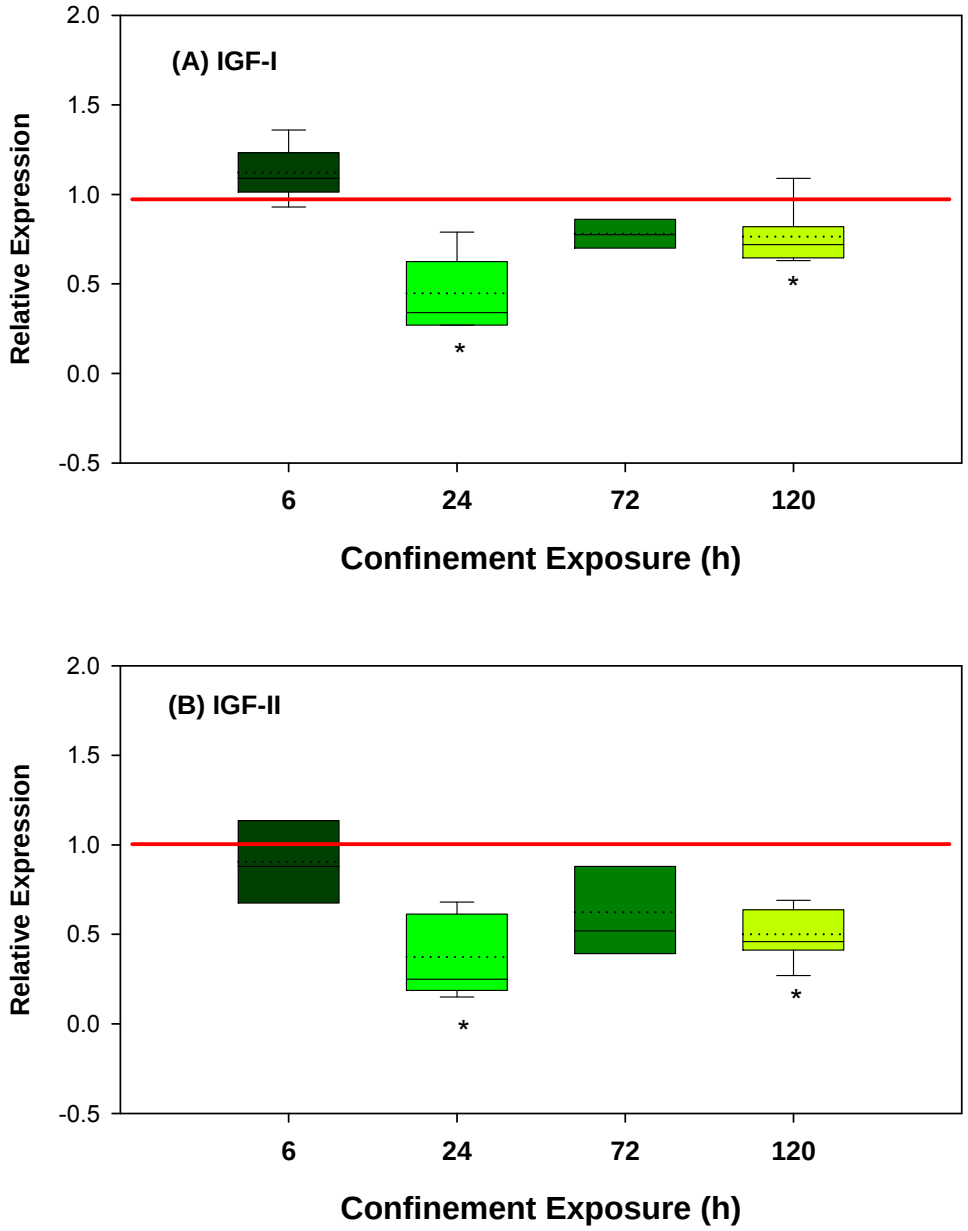


Figure 2. *Box-Whisker plot representing the relative gene expression of hepatic IGF-I and (A) and IGF-II (B) after confinement exposure. Inside box, the dotted line represents the mean value; continuous line is the median. At each sampling time, data were referred to values of non stressed fish (Student t-test; *, >0.05)*

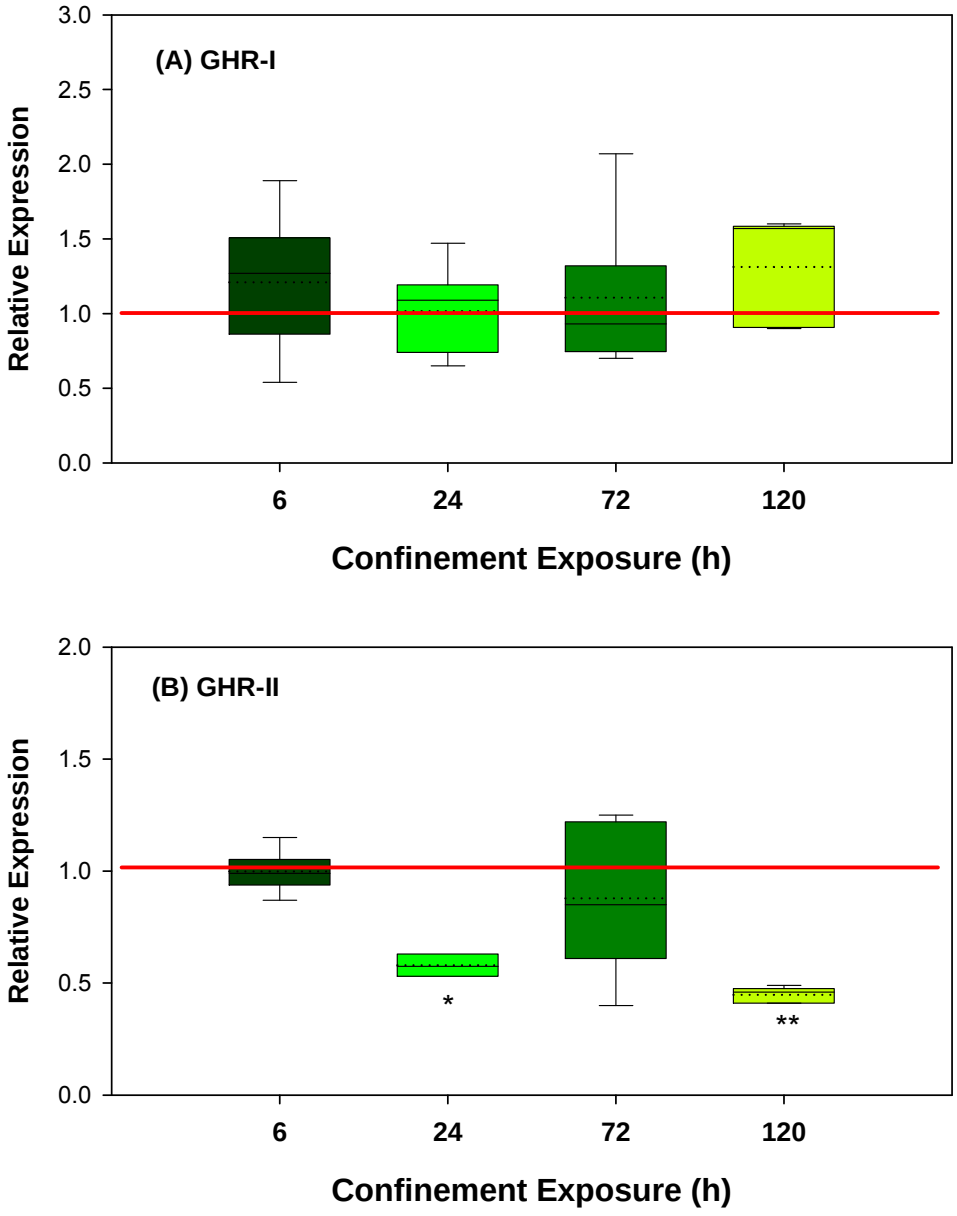


Figure 3. *Box-Whisker plot representing the relative gene expression of hepatic GHR-I (A) and GHR-II (B) after confinement exposure. Inside box, the dotted line represents the mean value; continuous line is the median. At each sampling time, data were referred to values of non stressed fish (Student t-test; *, >0.05: **, >0.01)*

Parasite infection

As reported in the first annual report, the degree of infection of the intestine was evaluated histologically and by parasite-specific PCRs. According to this combined diagnose, fish were classified as control (non-exposed), R-PAR (recipient fish exposed to the parasite and parasitized), and R-NonPAR (recipient fish exposed to the parasite, but not parasitized).

The expression profile of target genes [GRP-75, TNF, interleukin 1β (IL-1β), α2 macroglobuline (α2MG), glutathione reductase (GR), glutathione peroxidase 1 (GPX1), IGF-I, IGF-II, GHR-I and GHR-II] was then analyzed in the head kidney and intestine of R-PAR and R-NonPAR, using control fish as reference values.

Figure 4A shows the results of the head kidney expression of pro-inflammatory cytokines (TNF, IL-1β) anti-proteases (α2MG) and antioxidant related genes (GR, GPx, GRP-75) in R-PAR (A) and R-NonPAR (B). No significant changes were found in any of the genes of R-NonPAR, whereas only GPx was significantly down regulated in R-PAR. Figure 5A shows the expression profile of the same genes in the intestine, and interestingly GRP-75 and α2MG were significantly up-regulated, whereas pro-inflammatory cytokines and GPx were down regulated in R-PAR. No changes were found in the expression pattern of any of these genes when R-NonPAR was considered (Fig. 5B).

Concerning the expression of growth and immuno-stimulatory related factors (see Calduch-Giner *et al.*, 1995; Calduch-Giner *et al.*, 1997), IGF-I and GHR-I were significantly down regulated in parasitized recipient fish (Fig. 6A). An opposite pattern was found in non parasitized recipient fish (Fig. 6B) whereas the expression of both GHR-II and IGF-II remained unaltered in parasitized and non parasitized recipient fish, which provides additional supports for a different regulation and cell function of each GHR and IGF subtype.

Taken all this together, the significant up-regulation of α2MG in the intestine (but not in head kidney) of parasitized fish suggests a role of this versatile anti-protease to counteract parasite proteases at the local level of infection (intestine). At the same time, the ability to successfully combat parasitic infections requires that the host mounts an effective inflammatory response against the parasite. However dysregulated expression of pro-inflammatory cytokines can result in excessive tissue injury. Thus, several murine models indicate that the initial tissue pro-inflammatory response of sepsis is followed by an anti-inflammatory response. Accordingly, after long-term exposure to the parasite, parasitized sea bream are probably facing this anti-inflammatory phase in an attempt to prevent further tissue damage by ROS, generated by activated leukocytes during the pro-inflammatory phase. This hypothesis is in accordance with changes on the expression of GPx and GRP-75. However, more detailed studies with other chaperones and antioxidant genes are needed to better understand the complex regulatory network governing inflammatory and anti-inflammatory processes.

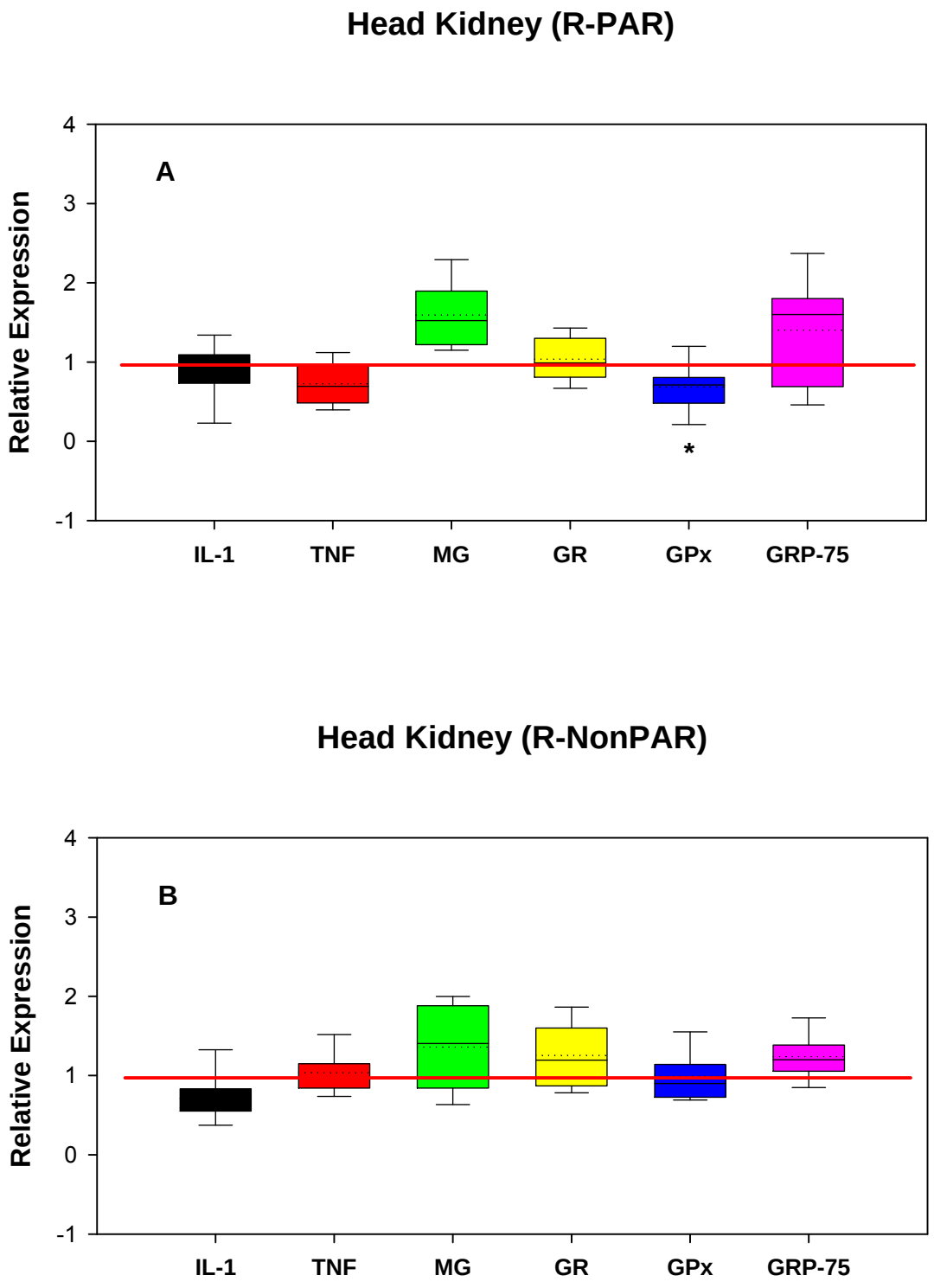


Figure 4. *Box-Whisker plot representing the relative gene expression of target genes in the head kidney of parasitized (A) and non parasitized recipient fish (B). Inside box, the dotted line represents the mean value; continuous line is the median. Data were referred to values of control fish (non exposed and non infected fish) (Student t-test; *, >0.05)*

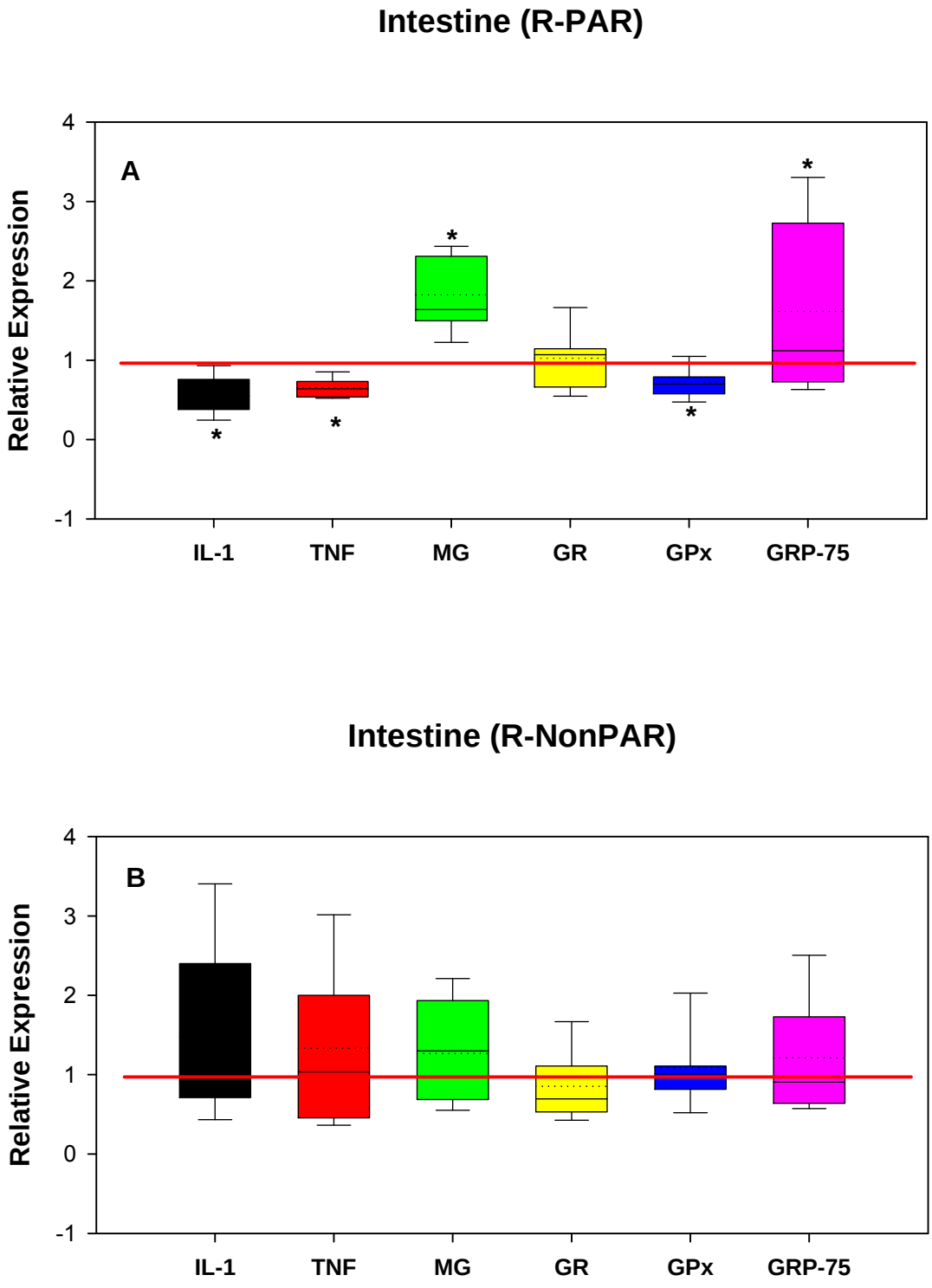


Figure 5. *Box-Whisker plot representing the relative gene expression of target genes in parasitized (A) and non parasitized recipient fish (B). Inside box, the dotted line represents the mean value; continuous line is the median. Data were referred to values of control fish (non exposed and non infected fish) (Student t-test; *, >0.05)*

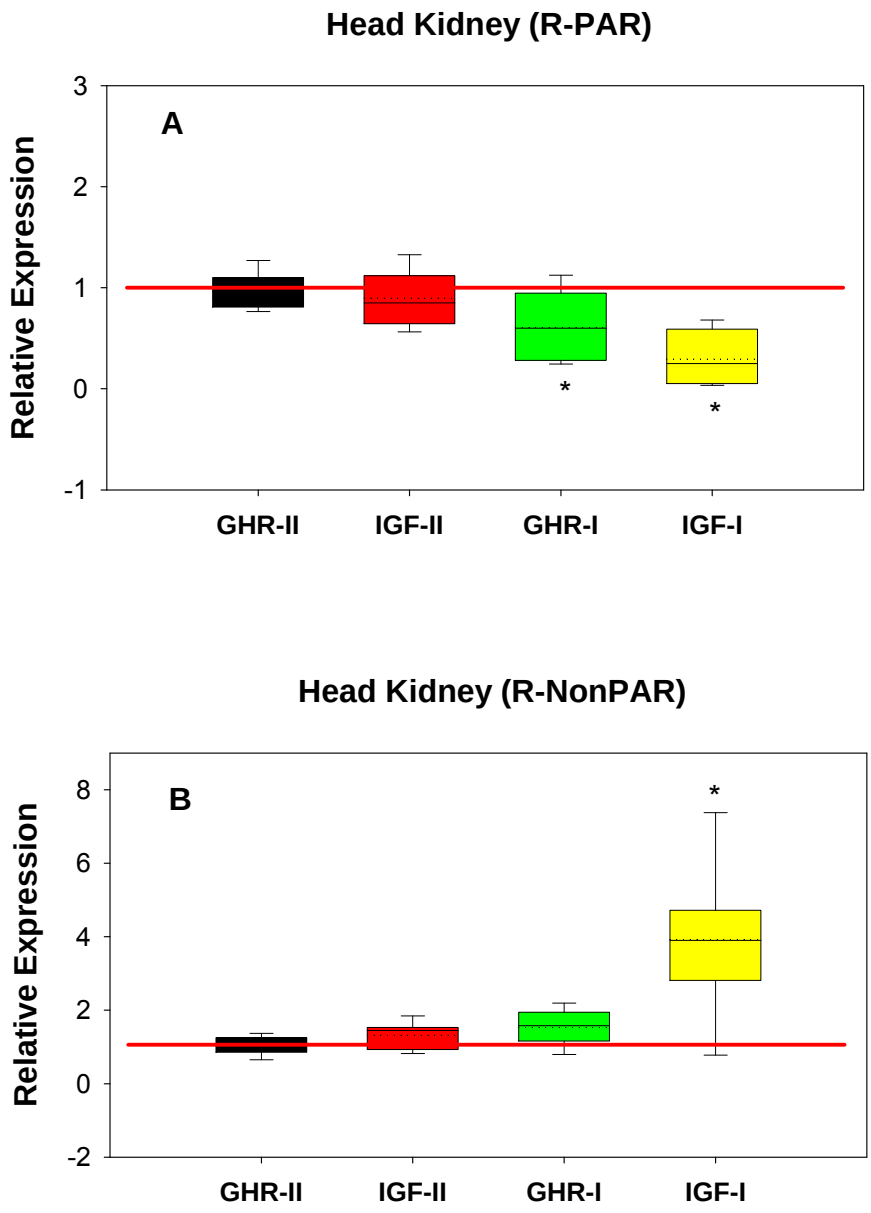


Figure 6. *Box-Whisker plot representing the relative gene expression of GHRs and IGFs in the head kidney of parasitized (A) and non parasitized recipient fish (B). Inside box, the dotted line represents the mean value; continuous line is the median. Data were referred to values of control fish (non exposed and non infected fish) (Student t-test; *, >0.05)*

References

Calduch-Giner *et al.* (2003) *Comp. Biochem. Physiol*. **B** 136: 1-13.

Kau *et al*. (2002) *Exp. Gerontol*. **37**: 1157-1164.

Coschigano *et al*. (2003) *Endocrinology* **144**: 3799-3810.

Holzenberger *et al*. (2003) *Nature* **421**: 182-187.

Rakoczy (2003) Mechanisms of Ageing and Development **124**: 1013-1024.

Calduch-Giner *et al. (*1995*) J. Endocrinol.* **146**: 459-467.

Calduch-Giner *et al.* (1997*) Cell Tissue Res* **287**: 535-540.

Deviations from the project workprogram

Delay of the delivery of microarray indiuced consequently a delay in the analysis of gene expression profiles in the various in vivo experiments carried out by the project. The amin consequence of such delay would be to hamper the progress of the next WP, characterization of SNP in stress or pathogen-regulated genes in trout and oyster. To solve this problem, we have organized a tied schedule in the timing of these analysis in trout and oyster: microarray analysis will carried out immediately after production of the microarrays by Rennes (trout oligos microarrays) or MPI (oyster microarrays). This task has already started for trout and will be done by the end of january 2007. For oyster, this task will be done in April.

List of deliverables

Code	Title	Due date	Actual/foreseen achievement date
3.3	In vivo exposure to stressor and pathogen (extrem families of seabass, seabream)	21	October 2006
3.4	RNA extraction (seabass, seabream)	23	October 2006
3.5	All gene expression profiles for the 4 species	27	October 2007

WP04: SNP : Single nucleotide polymorphism (trout, oyster)

Start date or starting event	1					
Activity type [1]	RTD/Innovation					
Participants	CO01	CO01.1	CR02	CR04	CR05	CR10
Person Months	9.7	26.5	2.15	5	12	11.38

Objectives

The objective of this workpackage is to develop Single Nucleotide Polymorphisms (SNPs) in genes or ESTs (Expressed Sequences Tags), in trout and oyster, to be mapped in the QTL analysis (WP5 and 11). The analysis of these SNPs are to be carried out on:

- sequenced promoter regions provided by Partners CO01, CR02, CR04 (T04.01),
- the candidate genes previously characterised (T04.02a),
- the new ESTs developed in WP1 and WP3 (up or down regulated genes) (T04.02b).

During the second year of the project, our work still focused on the candidate genes previously characterised (T04.02a) as the newly isolated ESTs developed in WP1 and WP3 (T04.02b) are still not yet available. The implication of this delay on our WP will be detailed in this report.

The partners involved in this task (T04.02b) are:

- CO01.1 : Institut National de la Recherche Agronomique (INRA CRJJ)
- CR05 : Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer La Tremblade)
- CR10 : Plymouth Marine Laboratory (PML)

INRA working on trout and Ifremer and PML on oyster.

For oyster, at PML, progress in the second year of the Aquafirst project has been limited to 7 months due to illness and the subsequent tragic death of the PDRA employed on the project. PML has however, now covered this position and is working to complete the deliverables as soon as possible.
PML scientists established that faster growth and longer survival in individual shellfish result at least in part from slower intensities with which proteins are renewed and replaced within the body as a whole. PMLs focus for SNP investigation is therefore on genes that affect protein turnover, in particular proteolytic enzymes, as it is hypothesized that variation in these genes may be responsible for resistance or susceptibility factors at play during summer mortality in the oyster.
Genes previously isolated at PML and sequences of genes from the Gigas Base database (http://www.Ifremer.fr/GigasBase/) have been selected for their putative role in protein turnover, metabolism or stress. For all of these gene sequences primers have been designed and tested on oyster genomic DNA to assess size and purity of the amplicon. For those showing a suitable amplicon the product was subsequently sequenced to confirm its identity. Of the original primers designed approximately 20% resulted in a clean amplicon of the correct gene ranging in size from 300 – 850 base pairs. We are currently redesigning primers to the remaining gene sequences to repeat this process: see advances in Table 1 where ✓ means "achieved" and × "underway".

Amplification has been successful for leucine amino peptidase (LAP), aminopeptidase N (ApN), alanine aminotransferase (AmT), aspartate aminotransferase precursor (Asp) and arginase (Arg). Successful amplification of glutamate dehydrogenase (GDH) resulted in amplification of a universal stress protein rather than the specific gene.

Table 1: Progress of SNP investigation on selected genes

Gene	Primers designed	Product amplified (test genomic)	Product sequenced	F0 screened	SNPs identified
Leucine amino peptidase	✓	✓	✓	×	×
Alanine amino transferase	✓	✓	✓	✓	✓
Amino peptidase N	✓	✓	✓	✓	×
Phosphoglucose isomerase	✓	×	×	×	×
Glutamate dehydrogenase	✓	×	×	×	×
Aldolase	✓	×	×	×	×
Phosphogluconate dehydrogenase	✓	×	×	×	×
Aspartate aminotransferase precursor	✓	✓	✓	✓	×
Cathepsin L procathepsin	✓	×	×	×	×
Nicotinamide nucleotide transhydrogenase	✓	×	×	×	×
Phosphorylase kinase	✓	×	×	×	×
Malate dehydrogenase	✓	×	×	×	×
Superoxide dismutase	✓	×	×	×	×
Arginase	✓	✓	✓	✓	×
Dual-specificity tyrosine phosphatase	✓	×	×	×	×
Translocase mt membrane	✓	×	×	×	×
Glyoxylate reductase	✓	×	×	×	×
Ornithine decarboxylase antizyme	✓	×	×	×	×
Ornithine decarboxylase	✓	×	×	×	×
Amino acid transport protein	✓	×	×	×	×
Transglutaminse	✓	×	×	×	×
Amino acid permease	✓	×	×	×	×
M2 pyruvate kinase	✓	×	×	×	×

Screening has begun on the 24 F0 parents for the 5 successfully amplified genes. For alanine amino transferase SNP identification is complete. Sequencing has been performed in both directions on the previously extracted genomic DNA of the F0 parents. All polymorphic sites were manually double-checked by comparing the forward and reverse chromatograph reactions and the sequences aligned using DNAstar software. All SNPs and indels were recorded but only those seen in more than one individual were recorded as a true SNP, singleton markers were not included due to the possibility of their resulting from PCR replication errors. The SNP positions and type for alanine amino transferase are shown at the end of the oyster part of Deliverable D04.01.

For oyster, at Ifremer, we continued our effort on SNPs identification:

	Year 1	Year 2
Number of ESTs studied	80	80
Number amplified in the F0 grandparents	29	41
Total number of sequences	624	984
Number of SNPs	167	290
Monomorph ESTs	7.7%	3.2%
Level of polymorphism / 100 bp	2.2	1.97

Although we worked with a total of 56 ESTs, the sequences of only 41 were completely corrected and taken into account as some ESTs turned out to be ribosomal genes, and others could be amplified but were never correctly sequenced.

We focused on the DNA polymorphism and codon usage bias in this set of 41 nuclear loci for the 24 F0 parents of families. A total of 290 single nucleotide polymorphisms (SNPs) were detected, 76 of which were localised in exons and 214 in non-coding regions (Fig 1). Our results revealed an astonishing level of DNA polymorphism in oysters. Non-synonymous mutations represent 10% of markers, and 16% are synonymous mutations in coding DNA. Average density of SNPs was estimated to be among the highest levels reported to date with one SNP every 61 bp in coding regions and one every 41 bp in non-coding regions. As an example, in humans one SNP is found every 1kb and in soybean one SNP is found every 610bp. Non-synonymous mutations contributed substantially to the polymorphism observed in coding regions. The ratio of non-synonymous to synonymous polymorphism was 1.5 on average. We also examined codon usage bias. The table of optimal codons use in the Pacific oyster was deduced from the analysis of an EST dataset. As recently observed in a few other taxa, we found a significant negative relationship between codon bias and non-synonymous diversity suggesting correlated selective constraints on synonymous and non-synonymous mutations

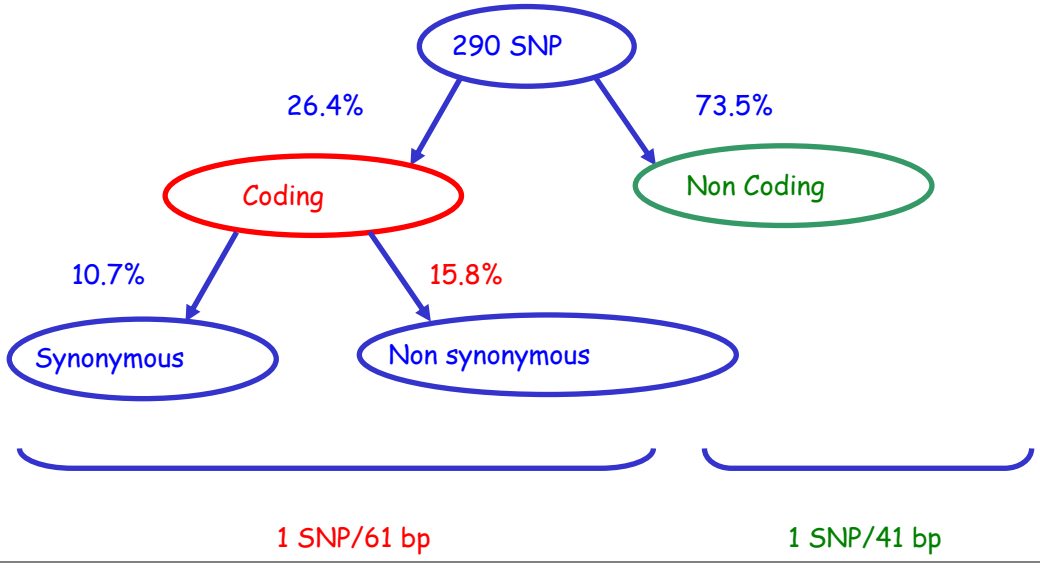


Fig 1. Characterisation of newly developed SNP from a set of 41 ESTs

With the identification of SNP markers, we are now planning the best way to genotype these markers on the F1 and F2 progenies. We plan to do this work during the first semester of 2007 with the use of Taqman or MS based (Sequenome, Good assay) genotyping methods in collaboration with the Max Planck Institute genotyping platform in Berlin.

In trout, we have to find SNPs polymorphisms on candidate genes provided by partners 1 and 2. We are looking for genes differentially expressed between the two lines selected for their different response to stress: HR (highly responsive) and LR (low responsive). As there is a delay for the production of this data, sequences won't be available before January 2007. Therefore we focused our work on sequences provided by partner 1, issued from the STRESSGENE project to estimate a success rate of developing SNPs markers. For 124 genes or ESTs we defined 167 pairs of primers using primer3 software. For 64% of them primer design was successful (1 clear-cut band in agarose).
We sequenced 36 of these genes. Due to the salmonids genome duplication, the observed variability on a chromatogram can be due either to real allelic polymorphism or to the superposition of two lightly different sequences. To solve this problem, we performed the sequencing on homozygous individuals provided by partner 1.1. Any polymorphism observed could be then attributed to the presence of two sequences (duplication, family of gene...). Results are summarised in the table below. Description of the ESTs was given using sigenae tools (http://www.inra.fr/agenae/SIGENAE.html).

We obtained good quality sequence for 60% of the pair of primers, representing 70% of the studied genes. We sorted sequences into different categories :

- unreadable: due to technical problems or real presence of several sequences,
- unreadable due to the presence of two overlying sequences making it impossible to work with,
- duplicated, partly readable: only some base pairs exhibit duplication, therefore possible to look at allelic polymorphism by comparing several individuals,
- Usable: suitable to analyse polymorphism in parents of the QTL families.

The 60% of good results could still be improved, as some of the duplicated sequences can exhibit allelic polymorphism.

Trout genes	Accession gene number	result on agarose gel	sequence	intron		Primers sequence
Cyclin-E binding protein 1	CT572154	ok	unreadeable			TCCATCTTGACTCCATTTCC
Human						AGAAGACCGTTGCTGTTTCC
Translocon-associated protein	CT571787	ok	2 sequences, unreadeabble			AGACGTGGCTGTAATAATTGG
						GGCATCAGAAGTGGTGTAGG

Transposable element Tc1	CR944126	ok	2 sequences, unreadeabble			TTCATACAGACGTGACACTTGG
						GCAATCCGGGGAGAAAGG
Oncorhynchus mykiss StAR mRNA for steroidogenic acute regulatory protein	AB047032	ok	low quality sequences			ATATGTGAAGCAGGGAGAGG
						GGTGAACTTGGTCTTGTTGG
		ok	low quality sequences			ATGAAGGGGAGAGACTAGGG
						GTGACTTGGGTGGTTATTGG
COLI1_ONCMY (Q04617) Corticotropin-lipotropin A precursor	CR943829	ok	ok			ATGGAGGGTTCATGAAAAGC
						CAGAGGACAGGGCTTTTAGC
	CT565374	ok	ok			GTTTGCTTAGCGAGGAAGG
						CCCCACTAGAAGACTCAAAAGG
COLI2_ONCMY (Q04618) Corticotropin-lipotropin B precursor	CR943812	ok	ok			AACTGCTATCAAGCGCTACG
						TATTGCGGAGCTATAATTCG
TYBB_ONCMY (P26352) Thymosin beta-12	CR943443	ok	ok			TCACCTCGTAGCTGTGTAACC
						ATCACACCCTTGCACTTCC
	CU068526	ok	low quality sequences			GGAGAAACGGAGCACAGG
						TCAACTAAACACTGGGACAGG
G6PI_HUMAN (P06744) Glucose-6-phosphate isomerase (	CR943446	ok	low quality sequences			TCCCAAAAGTGATTGATTCC
						TCTCTTCTTCATGGCTGACC
KCRB_HUMAN (P12277) Creatine kinase, B chain	CT565788	ok	ok			AGCAAGCACGAGAAGTTTGG
						GGGGAGATACTGGTGAGAGG
ZSWM4_HUMAN (Q9H7M6) Zinc finger SWIM domain containing protein 4	CR944130	ok	ok			GATGCTTTATGGTTGCTTGG
						AACCGCTAAATTGGAAAAGG
1433B_MOUSE (Q9CQV8) 14-3-3 protein beta/alpha (Protein kinase C inhibitor protein-1)	CR943504	ok	low quality sequences			CCTCCCTTCCTTTTGATTCC
						AATTGCACTGCTACTCAAACC
K0830_HUMAN (O94919) Probable endonuclease KIAA0830 precursor	CR943415	ok	ok			GTGAACATCCCATCTCACC
						TCAATATCGTGCATTTGTCC
HG2A_RAT (P10247) H-2 class II histocompatibility antigen, gamma chain (MHC class II associated invariant chain)	CT572519	ok	ok			TTTTATCGGTTTACCTTTCC
						CACAACACCCAGACTTTTCC
SUCB1_HUMAN (Q9P2R7) Succinyl-CoA ligase [ADP-forming] beta-chain, mitochondrial precursor	CR943486	ok	ok			TTCTGAGATTGTGTCGTTGG
						GGGAGGAAAGTCGAGATTAGG
GTP_CRIMI (P47954) Glutathione S-transferase	CT571763	ok	ok			AAGACTGGTTTTCTCGTTGG
						AGCGAAGGTAAATGGATGG
PABP1_XENLA (P20965) Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1)	CU070913		low quality sequences			TGAGGACTGATACTGGAAGAGG
						GCTTCACCGAAGAAAATGG
UPAR_RAT (P49616) Urokinase plasminogen activator surface receptor, GPI-anchored form precursor	CT571745	ok	ok			ACCACCTCCATCGTAACAGG
						CAATGCCAATGATTTGAAGC
ELOV4_MACFA (Q95K73) Elongation of very long chain fatty acids 4 protein (QtrA-14469)	CR944267	ok	ok			TTATTGATGGCCTTGACTGG
						CAGGCAAATTAGACAAGTGG
NOG1_HUMAN (Q9BZE4) Nucleolar GTP-binding protein 1	CT568820	ok	ok			CACCCACAGTCATAGCTTCC
						CAATTTAGCATTTAATCAGTTCC
Q9PTB4 GAP-associated phosphoprotein p62 (Fragment).	CT962993	ok	ok			GCGAGGCAGGATAAGAGG
						AACTGTCAATGATGCTGTGG
		ok	ok			TTTGCAGAAACTAACATTCC
						CATGTCCAAATTCTTGTAGAACC
		none				GACAACTATTACAGTCAACAACC
						AAGTGAGTTACAATGGGAGACC
calcium-binding protein	CT962658	ok	duplicated but partly readeable			ACAGGTCCAGCAGGGTATGG
human rabbit						TGCTGTTCTGTGGAGAGTGG
S10I_ICTPU (Q91061) Ictacalcin		ok	low quality sequences			CTGAGCTTGGAGAGATGATGG
						TTCACCTGTCTGTCTATGTGTGG
	BX084078	ok	duplicated but partly readeable			TGGTAGGTGGTATGCTGTGG
RETB2_ONCMY (P24775) Plasma retinol-binding protein II (PRBP-II)						CTCTGCAGGAGTAGTGGATGG
		ok	ok			TTGTCACCGACAAGAAAAAGG
						AATCATTTGGTTGGTTGATGG
CO3_ONCMY Complement C3-1 [Contains:						

C3a anaphylatoxin] (Fragment)	CT563394	ok	2 sequences, unreadeabble			CAACCAGATCACAACCTTCG
						AGGCAGGGTCATTTTAGTGC
Complement C3-1 [Fragment]	BX083340	ok	ok			ACAAGGTTCCAGAGCACACC
						GACAAGCCACTCCTTTACCG
		ok	2 sequences, unreadeabble			AGGTCAGCTGGTGAAAGTGG
						AACACCCCCATATTGTCTGC
		ok	ok			GATATTGGCCTGGACAAACG
						TCTGATCCCCTCACGTTACC
		ok	ok			TGCGACCATGTCTATTCTGG
						CTGGCTCACCTTTCTTCTGC
MAPE3_HUMAN (Q9UPY8) Microtubule-associated protein RP/EB family member 3 (Protein EB3)		ok	low quality sequences			GAG CAA TTT GAT TTA TCT GTT GG
						GAG GAA CTC AGT CTC TAG TTT GG
		none				ACA ACT TCA AAC TTC TAC AGA CC
						TAA CCA AGC ACG CAG ACC
PDX1_HUMAN (Q06830) Peroxiredoxin 1	BX073633	2 bands				TCA TCT GGA AAC GCT AAG ATA GG
						CTG GAG ATA GAC TGG GTG AGG
		ok	ok			CAG GGC ATC GCT TAC AGG
						AGC GTG GGT CAG AAA TGG
ABCG2_HUMAN (Q9UNQ0) ATP-binding cassette	BX082639	ok	ok			ATG ATG GCG ACG ATG ACC
						GGT ACG GAC TAA CAG CTC TCC
		2 bands				GAG GGA TGC TGA GGT ACT GG
						CAT CGT CTT CAG CTG TGT GG
HCE1_ORYLA (P31580) High choriolytic enzyme 1 precursor (EC 3.4.24.67) (Hatching enzyme zinc-protease HCE 1 subunit)	BX084352	none				CGA ACT GCA GTC AAA ACA CG
						CAA CGC ATC AAT CAT CAT CC
		ok	ok			TTG AAA GGA GCT CAG GAA CC
						GCA TCA GCA GCA ATA TAC CC
		ok	ok			AGG GTA TAT TGC TGC TGA TGC
						TGT AGT GCA AAC CCA TAT GAC C
Oncorhynchus mykiss strain OSU-142 T-cell surface glycoprotein CD8 alpha precursor	AF178055	ok	ok			CTGAGAGAGACGCAAGATGG
						TCTTTGGTTTCATTTACAGACC
		ok	ok			GATGGATTTCAACGATATTGC
						TCAAAATGCAATTACTGAAACG
Oncorhynchus mykiss clone OSU natural killer cell enhancement factor (Nkef) gene	AF250193	ok	ok			CTCCAAGCAGCAGTAAGACG
						CTGTTCCTCATTTCAGACTATCC
		multiple band				ATGTCAAAGTGGCCTACACC
						CGCATCAATCAAAGAGTTCC
		ok	ok			GTCGGACAAACTGAGAAACG
						TAGACCACTTCGCCACTAGG
		ok	ok			ACCTGTTGATTAGGTGTTGG
						TAGACCCCAGGAAAGTTAGC
Oncorhynchus mykiss tnfa gene for tumor necrosis factor alpha (TNFa)	AJ278085	none				ACACACTGGGCTCTTCTTCG
						CCTTTGAGATGGCTCAGTTACC
		ok	two sequences			GCT ACA GGG GAA CCA AAT CC
						GGT CTC AGT CCA CAG TTT GTC C
		ok	duplicated			TGC ACT GTG ATG AGA GAG AGG
						GAG AAT GGA CAA TCA TGT GTG G
		ok	duplicated			TGAAGGGGACAAACTGTGG
						CTTTGAGATGGCTCAGTTACC
Oncorhynchus mykiss iNOS/NOS2 gene for inducible nitric oxide synthase	AJ295231	ok	ok			CTCCATGCTAAACGGTGCC
						GAGATACTGCAAGGAGAAAAG C
		ok	ok			GCT CTC ACC GCT CTC AAG G
						ACC AAA ATG GTG GAC CGG
		ok	ok			TGC TTC AGG ATG ATG AAA CC
						CTT TTG ATG TGC ATG GTT GG
promoter IL1 beta 1	AJ298294	ok	ok			GACAATGACCATAGGAGTTGG
						TGTGATGTACTGCTGAACCC

Oncorhynchus mykiss iip2 gene for interferon inducible protein 2	AJ313031	OK	low quality sequences			GGCAACCCATTATGTCTCGG
						AAAGCAGCGTGCAGTCTTCC
		none	two sequences			GACTTCACCATGCTCAAAGG
						TGCAAAGATGACCTTCATGG
		none				AGAGTTCGCCATCCATTACG
						CACACACGTTTGTCCACTCG
		ok	ok			TGT GGT TTG GTT GAG TTA GGG
						AGA CCA ACT GGG TGA CAA GG
		ok	ok			CGT TTT GCC TTC ACC TTT CC
						CAT GCC CAA CTT CAA AAT CC

Until last month, the choice of the trout families for QTL studies was not completely defined, depending on survival of these families. For these reasons, no polymorphism studies on these parents have been made. Consequently, no SNPs have been characterised in the families however, the tools (see table above) are now ready to perform this task. Furthermore, the choice of the families has now been done and fin clip of the family QTL is being carried out in December by partner3. The SNPs will then be characterised in these individuals in early 2007 and genotyping will be subsequently performed.

Deviations from the project workprogram

Finally, deliverable D04.01 "Sequences (and primers) of SNPs from previously characterised genes" planned to be delivered on time in Month 18 is given just below with a total of **636 SNPs for oyster** but none for trout for the reasons detailed above.
As sequences of new ESTs produced in the project will be delivered later than initially planned, this will subsequently delay our delivery of D04.02 "Sequences (and primers) of SNPs from new ESTs". Furthermore it was concluded in the meeting held in Heraklion (November 2006) that it would not be possible to study the "sequenced promoter regions" as Partners CO01, CR02, CR04 would not be able to provide them to us as initially planned in the project. However the partners involved in WP4 will try to characterise even more SNPs in the new ESTs provided by WP1 and WP3 at the beginning of 2007.

List of deliverables

Code	Title	Due date	Actual/foreseen achievement date
4.1	Sequences (primers) of SNP from previously charactcerized genes	18	October 2006
4.2	Sequences (primers) of SNP from new EST	30	end 2007
4.3	Table of the charcaterisation of the SNP (insertion, deletion, sustitution).	30	End 2007

WP05: Characterisation of QTLs related to stress and disease resistance in trout and summer mortality in oyster

Start date or starting event			1						
Activity type [1]			RTD/Innovation						
Participants	CO01	CO01.1	CR02	CR03	CR04	CR05	CR08	CR09	CR10
Person Months	22.7	23.8	14.88	9.93	20	21	16	4	5.27

Objectives

The objective of WP05 is to identify associations between phenotypic traits relevant for stress or diseases resistance and genetic markers that stand for chromosomal segments expected to carry QTLs (Quantitative Trait Loci) of significant effect on the variability of the traits. The identified genetic polymorphisms will be further used in operational genetic protocols for selective breeding.

Two species, rainbow trout and Pacific oyster are involved in WP05.

The WP is organised in several tasks:

Task 05.1: Production of trout and oyster families segregating for traits related to stress and disease.
In both species, involved partners had previously selected lines that are divergent for traits related to response to stress (blood cortisol after confinement stress in trout, *Partner CR03*, and summer mortality in oyster, *Partner CR05*). This allowed designing QTL experiments based on F2 families. Because of a two years generation interval in trout and oyster, the F1 parents necessary for the production of F2 families had been produced before the Aquafirst project has started.

Task 05.2: Phenotyping F2 offspring for traits relevant for stress and disease resistance.
Main target traits are response to different stressors (confinement, salinity) and infectious disease in trout, and summer mortality in oyster.

Task 05.3: Genotyping of parents and offspring
Microsatellite markers and SNP (Single Nucleotide Polymorphism) markers are to be used in both species to perform a genome scan for QTL detection. Some of the markers will be developed in the Aquafirst project (WP04).

Task 05.4: Linkage analysis between phenotypic values and markers in order to map QTLs associated with the target traits (in connection with WP11)

Progress towards objectives

OYSTER

Task T05.1:

Power optimization of QTL mating design was performed during Year 1 by *Partners CR8* and *CR16* (Wageningen University, Roslin Institute) in order to implement a design with the higher statistical power (type, number and size of families) given the total number of individual oysters to be genotyped and phenotyped. A design including 5 segregating F2 families of more than 2000 individuals was recommended.

On this basis, F1 hybrid parents (crosses between parents from lines susceptible or resistant to summer mortality) were mated in February 2006 (month 16) to produced F2 progenies. Five different F2 families with 4 replicates each were produced by bi-parental crosses (20 crosses reared at hatchery of *Partner CR05*, La Tremblade)). Two control crosses (wild oysters) were used as a reference cross to measure the intensity of the mortality event. Larval settled on mid-March and the spat was grown up in the hatchery until the size of 2mm. Then, all oysters were transferred into the Ifremer Nursery of Bouin (Vendée, France) for a 3 months period for growth.

The final choice of one of the four replicated cross in every F2 family was based on the number and average size of settled larvae, success rate at metamorphosis and the fact that oyster should not be injured in any mortality event during the growth period in the nursery.

Task T05.2:

When all oysters measured 2 cm (early July, month 20), they were brought back to La Temblade to be individually tagged and sampled. For each of the 5 selected F2 families, 500 individuals were randomly sampled and stored at –20°c before any technical step (for further DNA extraction and analysis if necessary). Then, oysters of each family were distributed for phenotyping experiment.

Field test:

To test the response to summer period (dead/alive) of F2 progeny, 500 individuals of each F2 families were placed on the field on the reference test site of Perquis in the Marennes-Oléron basin. Oysters mortalities are recorded since the 70's on this site. In this experiment, summer mortality was checked every week between the 15th of July and the 22nd of September on F2 groups. As a result, no mortality (< 0.001) occurred during this particular experiment.

Laboratory test:

Within a three day period, 1000 randomly chosen oysters of each selected F2 families were individually tagged and placed into a raceway for the phenotyping experiment. A sample of untagged individuals (n=500) from the same families was placed into the same raceway as a control of tagging effect on survival.

Between the 10th of July when summer mortality began to occur and the 31st of August when the experiment was stopped, mortality was monitored daily by removing dying oysters. Herpes virus was detected in the dying oysters during this period. Each removed dying oyster was individually put in bag to avoid Herpes virus contamination from one sample to another, and they were stored at –20°c. The same protocol was applied to untagged oysters. Moreover, 30 living oysters were also sampled every day in stock bags of the 5 F2 families to get a reference for further Herpes Virus quantification and analysis.

As a result, summer mortality occurred in the laboratory conditions and appeared to be associated with the presence of Herpes virus. The 5 F2 families were differentially affected by this mortality event (from only 1.2% of tagged oysters to 67%, see Fig.1).

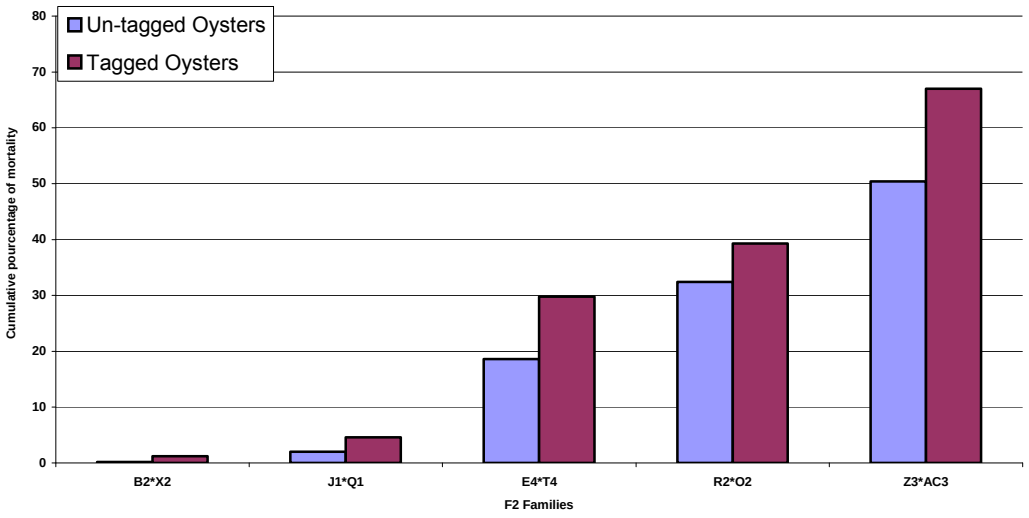


***Figure 1**: Effect of tagging and family origin on summer mortality F2 QTL families*

Dead and alive sampled individuals are now being qualified on the basis of the presence/absence of Herpes virus. A new method of Herpes Virus quantification based on a quantitative PCR has been developed in the Genetic and Pathology Laboratory (LGP) of the Ifremer station of La Tremblade. First results show a strong correlation between mortality rate and the number of Herpes virus DNA copy /mg of oyster tissue. As an example, very few copy (<1000) of Herpes Virus DNA were detected in oyster of the family exhibiting the lowest mortality record whereas F2 family, 104 to 106 of Herpes Virus DNA copies were detected in family with the highest mortality record. The analysis of the 3 remaining F2 families will be used to confirm this association.

Task T05.3:

Microsatellites genotyping on the 24 F0 and the 10 F1 individuals is still underway. Currently, 80 markers were tested and 80% gave us reliable information on the F0. F1 parents were genotyped for 30 of these markers.

The 3 F2 families in which fairly high mortality was recorded will be used for further genotyping. The two other families, in which almost no mortality was recorded, would not be informative (decision discussed with *Partners CR8* and *CR16*). Selective genotyping will be performed (150 early died individuals and 150 that survived until the end of sampling). Currently, DNA extractions are underway (300 individuals X 3 F2 families) and genotyping with microsatellites markers will begin in the next coming weeks.

Genotyping of SNP markers (produced in WP4) is planned during the first semester of 2007 (before month 32). We plan to do this work with the use of Taqman or MS based applications (Sequenome, Good assay) genotyping methods in collaboration with the Max Planck Institute genotyping platform in Berlin.

TROUT

Task T05.1:

As for oyster, QTL mating design has been discussed during Year 1 (same Partners) in order to implement a design with the higher statistical power (type, number and size of families) given the total number of fish to be genotyped and phenotyped (n=1000). Five to seven F2 families (150-200 progeny each) appeared as the best design (WP11).

F2 families were produced in January 2006, from F1 breeders maintained by *Partner CR03* since January 2004. In order to provide information about the state of maturity of the F1 fish, 350 blood samples were collected from fish from 18 families. Of these, only 22 fish in total, from 10 families, were found to be positive for vitellogenin, indicating a possible maturing female. Finally, only 19 females F1 breeders spawned. They were crossed with F1 unrelated males to produce 19 F2 families. In order to anticipate on poor gamete quality and low survival of progeny of those young females, 14 back-crosses were also carried out (3 years old females from pure parental lines x F1 males). All groups arising from this exercise have subsequently been reared in Partner CR03's facilities until October 2006 (month 24), when both number and mean weight of each remaining family were recorded. Nine F2 families had enough fry (200 and more) to be further used, among which five were chosen to produce a QTL design that fits the optimum one (5 F2 families, 210 individuals each). Grand parental origin was considered to choose among families: when possible, families from the most selected grand-parents were preferred. Attention was paid to minimize the number of common grand-parents. The number of informative microsatellite markers was used as a complementary criterion (see T05.3).

Family	Number of fish	Male origin	Female origin	Weight (g)	Nb Markers
X3	451	HY1D	HY24	26.5	111
X4	622	HY1B	HY4	16.3	105
X8	237	HY1C	HY21	45.3	104
X14	353	HY1E	HY12	22.4	103
X17	419	HY13	HY10	40.1	118

***Table 1**: Origin, number and mean weight of the 5 F2 trout families as of October 2006, and the number of informative markers in each cross (for at least one parent)*

The 5 F2 families are being tagged (months 25-26), fin-clipped, weighted and distributed into 4 tanks of 50 fish per family in order to facilitate further records of phenotypes (blood sampling after confinement stress) (*Partner CR03*).

Task T05.2: This task will start in January 2007 (month 27).

The experimental design for phenotyping of traits related to confinement (*Partners CO.01, CR03, CR04)* and salinity stress (*Partners CO 01, CR04)* has not been modified.

Confinement challenges (1h confinement, at least 2 replicated challenges) will be performed, and blood cortisol and catecholamine will be measured after stress in all QTL F2 individuals.

Salinity challenges (24h at 29g/l, 2 replicated challenges) will be performed on the same individuals, and plasma osmotic pressure and ion levels will be measured after challenge. At the end of the second challenge, gill biopsy will be performed on a sub-sample of fish for an eQTL analysis that will be performed with additional founding.

Immune challenge (*Partner CR02*) meets with animal welfare regulation which prevents to evaluate the resistance as a binary trait (dead/survivor) as initially planned. Preliminary experiments are being performed by *Partner CR02* in order to circumvent this difficulty and try to find indirect criteria that predicts correctly the resistant/susceptible status of individuals.

Meat quality (*Partner CO 01*) will be measured at the end of year 3 in a sample of pure parental lines selected for cortisol response specifically produced by *Partner CR03*. The objective is to precise possible relationship between cortisol response and meat quality and to check how genetic selection for stress responsiveness would impact flesh quality. Measures will involved instrumental flesh characteristics (pH, colour, texture).

Task T05.3:

In order to prepare the genome-scan of trout families, a number of microsatellite markers were chosen among those used to build the genetic map of *Partner CO01.1* (Guyomard et al, BMC Genomics, in press). Three criteria were used for the first step of selection (quality of pattern, polymorphism in a reference synthetic strain and position on the map to ensure whole genome coverage) and 197 markers were chosen.

To refine the choice, the polymorphism of those markers was tested in the strain used in the QTL experiment (*Partner CO01.1*). At the time when tests were performed, QTL families were not known yet. Ten F2 crosses were 'pre-chosen' on the basis of interim counts of fish, with the hope that the information could be used later on, and the 20 parents and the grand-parents of those families were used to perform the tests on markers.
Sixty one markers had to be discarded because they were monomorphic in the whole set of individuals, or in both parents of the tested crosses. The 138 remaining makers have been sorted and tested to design multiplexes. Eight multiplexes (51 markers) will be genotyped by LABOGENA (subcontractor of *Partner CO01.1*). Remaining markers (71 + 16 duplicated) will be genotyped by *Partner CO01.1*.
The overall mean marker distance for genome scan of QTL families is close to 20cM. After correction for the fact that selected markers are not all informative in the 5 crosses, the actual mean distance between markers is around 25 cM.

The position of informative markers in each family of the 5 families finally selected is being checked. Specific markers may be added in each family if necessary (large genome area not covered by the set of markers).

Fin clips will be used for extraction of DNA in F2 individuals. They are to be dispatched to *Partner CO01.1* by *Partner CR03* before end of month 26 for immediate processing (DNA extraction and microsatellite genotyping).

Deviations from the project workprogram

OYSTER

Task T05.1: oysters exhibited unexpected survival on field during this summer, but experiment was duplicated, and mortality occurred in laboratory test. Yet, two families also exhibited almost no mortality, and only 3 QTL families (instead of 5) are finally available. Yet, the remaining statistical power of the design is enough to go on with those families (discussion with Partners of WP11).

Task T05.2: additional phenotyping effort has been decided in order to characterize families for presence/abundance of Herpes virus. This will allow a better description of the summer mortality recorded in this particular experiment.

TROUT

Task T05.1: the choice and tagging of the 5 families of the QTL design was postponed to month 26. There were two main reasons for doing so. Fish were born from young females, exhibited poor survival in early stages and did not grow fast. The additional growth period allowed to choose families only after the summer period (and thus to take into account mortalities that often occur during this period) and to start experiment with larger fish (increased tolerance of tags and blood sampling). As microsatellite markers had been tested for polymorphism in the parents during the same period, it was also possible to include this information in the final choice of families.

Task T05.2: because of the delay in tagging families, the first records of phenotypes (blood cortisol and catecholamine after a 1h confinement stress) have been rescheduled in early January 2007. This will have no impact on the rest of the experiment (all phenotypes recorded by May-June 2007)

Task T05.3: in the project workprogramme, trout genome-scan in QTL families was to be performed with 100 microsatellite markers, and about 150 SNP designed from a list of genes differentially expressed after stressing trout groups selected for cortisol response to confinement stress or disease resistance (WP01 and WP03).

At the project meeting in May 2006, a new planning for the WP1/WP03 research activity was proposed, associated with some delay in the availability of the lists of differentially expressed in which SNP markers have to be designed to complete the set of markers used for genome-scan, and possibly, to some difficulty to get the required number of functional SNP for genotyping early enough.

To secure the number of markers for genome-scan and thus the power of detection of QTL, the number of microsatellite markers included in the genome scan was increased (additional effort was put on testing microsatellite polymorphism in F1 parents and in designing multiplexes, and 138 markers were finally retained, instead of 100 initially planned).

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D5.1	Production of F2 families (trout, oyster)	Month 16	Oyster: achieved (3 families, phenotyped and sampled for DNA) Trout: achieved (5 families tagging and DNA sampling on the way)

List of milestones

Code	title	Due date	Actual/foreseen achievement date
M05	Production of F2 families in trout and oyster for QTL mapping (phenotyping, genotyping)	36	36

WP06: QTL mapping of disease resistance in seabream

Start date or starting event		1			
Activity type [1]		RTD/Innovation			
Participants	CR14				
Person Months	33				

Objectives

- **1.** Perform a small-scale chromosome scan for the detection of QTL affecting resistance against a specific pathogen in sea bream.
  2. Estimate the variance contributed by these loci as well as the size of effect of particular alleles on the individual traits.

Progress towards objectives

During the first year, three different challenge experiments were carried out and a total of 3000 animals have been sampled. However, the first challenge showed excessive mortality distributed in a short period o f time (four days), therefore it was considered insufficiently informative for the presence of disease resistance. The second and third challenge were carried out quite late in the season with strong mortality associated to transport or other environmental difficulties. Both experiments showed a substantial proportion of surviving animals, and a wider distribution of mortality in time. Animals from the two experiment might pooled together, because the experimental setting was comparable. This should have provided the minimal number of family with sufficient sample size to proceed with genetic analysis. However, at the end of first year meeting, it was agreed to repeat the same challenge on a larger number of fish immediately after the start of sea bream spawning season (November 2005). Juvenile fish of 0.5-0.6 g were ready by the beginning of February 2006. The methodology for experimental infection was the same as in the previous experiments.

Methodology for experimental challenge
Since March 2005 in collaboration with the production manager of the fish farm "Valle Ca' Zuliani" (Monfalcone, Italy), we have started to produce experimental populations to be experimentally challenged with a pathogenic strain of *P. damselae piscicida*. Fertilized eggs originating from a single broodstock (50-60 broodfish) were collected and kept separate in a dedicated water tank. After hatching, larvae were raised following standard procedures, bringing them to metamorphosis. Juvenile sea breams were kept in a dedicated fish tank until reaching an average weight of 0.3-0.5 g. All animals were then transferred to the facility for experimental challenge (at the Instituto Zooprofilattico Sperimentale delle Venezie, Legnaro). Fishes were kept in two fish tanks with ricirculated, aerated sea water (1 cubic meter each). After 5-10 days of acclimation, sea breams were challenged with the pathogen by immersion as described below. Mortality was monitored twice a day for 20 days after challenge. Digital pictures were taken for dead fish, which were subsequently stored at -20°C for later DNA analysis.

Challenge 4
Approximately 3500 fish of 0.4-0.6g in weight were transferred into two fish tanks in the experimental facility. A relevant mortality was recorded immediately after transport from the fish farm to the lab (>250 fish). Mortality dropped to zero after few days of acclimation. At day 0 all fish were exposed by immersion in 50 l of aerated sea water containing 1x105 CFU of a highly virulent strain of P. damselae piscicida for 30 minutes. After pathogen exposure, all fish were returned to the fish tanks. After three days,

mortality was observed. The cause of mortality was assessed by histological evaluation of head-kidney, spleen, and liver of randomly chosen animals. Confirmation of diagnosis was carried out by PCR-RFLP analysis (Zappulli et al. 2005) of spleen from dead animals. All examined individuals showed the presence of *P. damselae piscicida* at high titre. Despite we used identical conditions as in challenge 2 and 3, where mortality was 70-75%, at the end of the experiment (20 days after challenge), approximately 3% of sea breams survived to the disease (>95% mortality). However, the observed mortality was well distributed along the experimental period, and after consultation with the coordinator and partners CR8 (Wageningen) and CR15 (Roslin Institute), it was decided that survival time (time to death) could be used as indicative of disease resistance. Mortality per day is reported for each tank in the following table

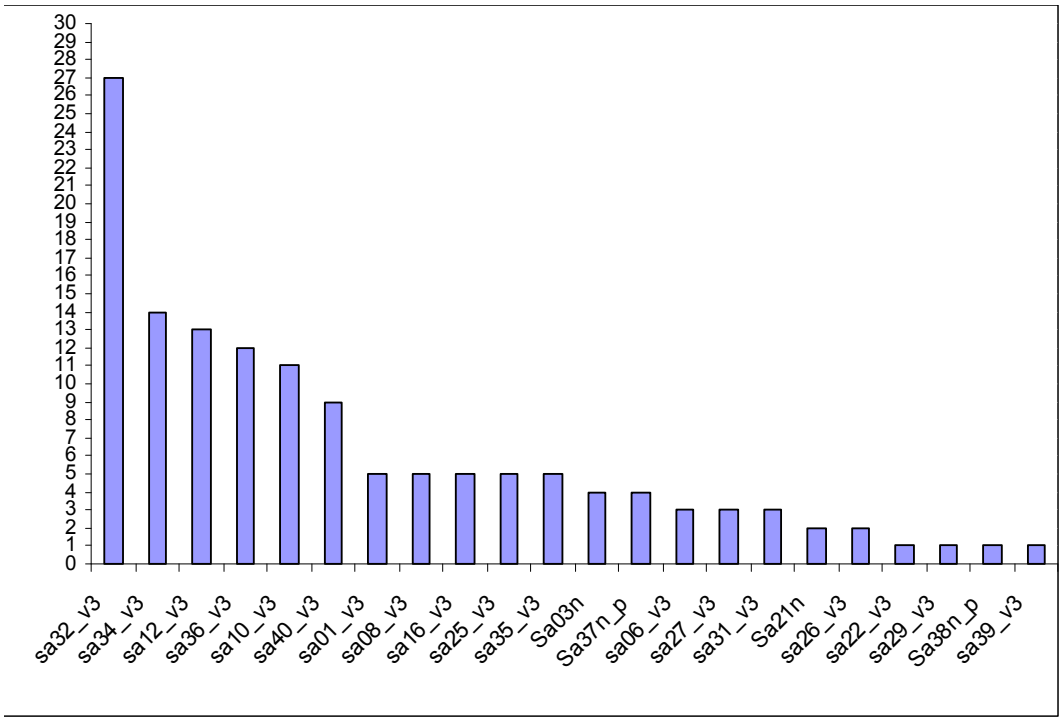
challenge	1d post ch	2d post ch	3d post ch	4d post ch	5d post ch	6d post ch	7d post ch
10/02/2006	11/02/2006	12/02/2006	13/02/2006	14/02/2006	15/02/2006	16/02/2006	17/02/2006
0	0	53	150	142	179	224	189
0	0	28	73	40	133	183	107

8d post ch	9d post ch	10d post ch	11d post ch	12d post ch	13d post ch	14d post ch
18/02/2006	19/02/2006	20/02/2006	21/02/2006	22/02/2006	23/02/2006	24/02/2006
183	137	198	187	137	59	106
138	90	193	169	105	36	50

15d post ch	16d post ch	17d post ch	18d post ch	end experiment
25/02/2006	26/02/2006	27/02/2006	28/02/2006	01/03/2006
pooled with 26/02	75	29	22	74
pooled with 26/02	36	22	8	22

A total of 3577 animals have been sampled, divided in two tanks (A and B). Disease trends were similar between the two tanks, which represent independent replicates. We decided to analyse at least 1500 animals from tank A (in total 2144 fish) and keep animals from tank B (1433 fish) as a back-up.
It was also suggested by partners Cr8 and CR15 to genotype for 6-9 microsatellite loci a limited number (100) of individuals of the experimental population together with all potential parents in the broodstock. This preliminary analysis was suggested in order to assess the number of contributing parents to the experimental population. In recent studies it has been shown that despite the high number of potential parents in a broodstock, during mass spawning the actual number of contributing parents might be quite low. We therefore proceeded following the above scheme. However, a serious delay in the genotyping process was caused by the fact that the broodstock had been only partially sampled (29 animals out of 54). Incomplete sampling was due to a misunderstanding with the production manager about the tank of origin for the experimental population. Such problem has been reported in the 18th month project meeting in Leuven. Complete sampling of the correct broodstock was expected by the end of August 2006, just before starting the new reproductive season. Unfortunately, due to internal problems in the fish farm, the sampling of all 54 animals of the broodstock has been completed only on the 20th November 2006. To partially reduce the effect of such delay, in agreement with the coordinator and partners CR8 and CR15, we decided to proceed with genotyping a larger number of individuals (250) as well as all available parents (29). This analysis was carried out for 9 microsatellite loci, and a specific computer program (PASOS ver. 1.0) was used to perform parentage analysis with incomplete sampling of parents. The obtained results showed that a sufficient number of

fish in the broodstock were contributing to the experimental population. In the following graph are reported on X-axis individual fish of the broodstock, and on the Y.axis the number of fish in the experimental population which are assigned to a specific parent fish.



Although there is a bias in the individual contribution to the progeny, 22 out of 29 parent fish contributed to the progeny. Based on these results, we decided to proceed with the genotyping of 1500 individuals from challenge 4.
At present (25 November 2006) for all samples (1536 fish) genomic DNA has been extracted. For 480 fish genotyping has been carried out. All fish (54) from the broodstock have been re-sampled, DNA has been extracted and genotypic data have been produced. It is expected that all the remaining fish (1000) will be genotyped by the end of the year 2006. Parentage assignment is expected to be completed by mid January 2007.

Deviations from the project workprogram

As already described in the first year report, it was originally planned to sample 1000-1500 sea bream individuals at a fish farm where enteromyxidiosis was reported to be enzootic in fish raised in sea cages. Sampled animals should have been part of the one year old population, in order to be more easily traced back to the corresponding broodstock of origin and to avoid that the number of families sampled were to high, reducing the sample size for each family. However, the prevalence of enteromyxidiosis in one year old animals was extremely low during year 2005. Sampling two years old animals was excluded because in each sea cage fish originating from 4-6 broodstocks were present. As mentioned above, this would have led to a very large number of sampled families, with only 5-10 individuals for each family. As demonstrated by simulation studies (presented at an *ad hoc* project meeting in Paris), at least five families of 50-70 individuals each are required for reliable QTL detection given the experimental conditions. To overcome the encountered problem, it was agreed during the kick-off meeting that a valid alternative might be to focus on a different pathology (**fish pasteurellosis**), that is amenable to experimental infection in smaller animals. **Fish pasteurellosis** is also the most relevant infectious disease in sea bream aquaculture. The causative agent, *Photobacterium damselae* subspecies *piscicida*, is a Gram-negative

bacterium belonging to the Vibrionaceae group. *P. damselae piscicida* can infect also other cultured species (e.g. seabass). The pathogen causes acute severe septicaemia, especially in juveniles. In the adults is often observed as chronic infection. During epizootic outbreaks mortality might reach 90% of the affected stock. Therapy is usually based on antibiotics, yet losses are usually high. Prevention of the disease is possible through rigid measures of disinfection. An experimental vaccine is available, although in the stages with highest susceptibility (juvenile seabream of 0.2-0.5 g) the protective effect of vaccination is limited in time.

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D06.01	Sampling 1000-1500 individuals from fish stocks of seabream naturally infected with enteromyxidiosis (now	12	16
D06.02	All target specimens genotyped for 6-9 SSLP markers, parentage analysis performed on all individuals	18	27

List of milestones

Code	title	Due date	Actual/foreseen achievement date
M9	QTL data for disease resistance / susceptibility	36	36

WP07: QTL mapping of the stress response in sea bass and sea bream

Start date or starting event		Month 1			
Activity type [1]		RTD/Innovation			
Participants	CCMAR (CR11)	KULeuven (CR12)	HCMR (CR13)	Nireus (CR17)	
Person Months	24	14	6	5.7	

Objectives

a) Perform a genome scan for the detection of QTL affecting the stress response in sea bass and sea bream.

b) Perform candidate gene linkage analysis for stress response in sea bream and sea bass.

c) Estimate the variance contributed by these loci as well as the size of effect of particular alleles on the individual traits.

Progress towards objectives

During the reporting period the following activities have been carried out:
1) Repeat the sea bream stress experiment
2) Measurement of blood parameters for sea bream and sea bass
3) Choice of markers for whole genome QTL scan

1) Repeat of sea bream stress experiment

In the last reporting period we described and provided deliverables for the stress experiments in sea bream and sea bass. However, soon after the sampling of sea bream it was discovered that the sampled population contained fish of different origins, added inadvertently by one of the fish farm employees. Since it is required that the experimental population is composed of siblings the experiment had to be repeated, this time at the Nafpaktos fish farm, where it could be more easily be monitored. A batch of 1 day spawning was used coming from a broodstock of 178 individuals. Fish were grown for 10 months and sampled in September 2005 following the methodology described in the previous report.

2) Measurement of blood parameters for sea bream and sea bass

The planned deliverable estimated that all of the circa 4000 blood samples of sea bream and sea bass would be analysed for cortisol by month 18. However, the requirement to repeat the experiment with the sea bream and a lengthy negotiation with the carrier to obtain the appropriate permissions for transport of samples in dry ice from Greece to Portugal delayed significantly the start of the analysis. Nevertheless, the analysis is now progressing well and it is expected to be completed by month 27.
Previous experiments using solvent extraction and chromatography have shown that the RIA is highly specific and only cross-reacts with one plasma component co-migrating with cortisol on thin-layer chromatography. In order to establish a swift and accurate procedure to analyse plasma cortisol we compared a procedure of straight dilution in radioimmunoassay assay buffer and dilution following by denaturing at 80ºCfor 1 h. The results (
Figure 1) indicate that for the two species, values of cortisol in non denatured plasma is ca. 10% lower than in denatured plasma, suggesting the presence of a corticosteroid binding protein. Despite being a small difference we decided that all the samples would be denatured.

Up to the date of this report 449 sea bass samples have been analysed for cortisol yielding the data distribution pictured in **Figure 2**. The results show a high variability and within the range expected for the stressor applied. It is estimated that the analysis of all the sea bass plasmas will be completed by the end of December 2006 and the sea bream samples 2 months later.

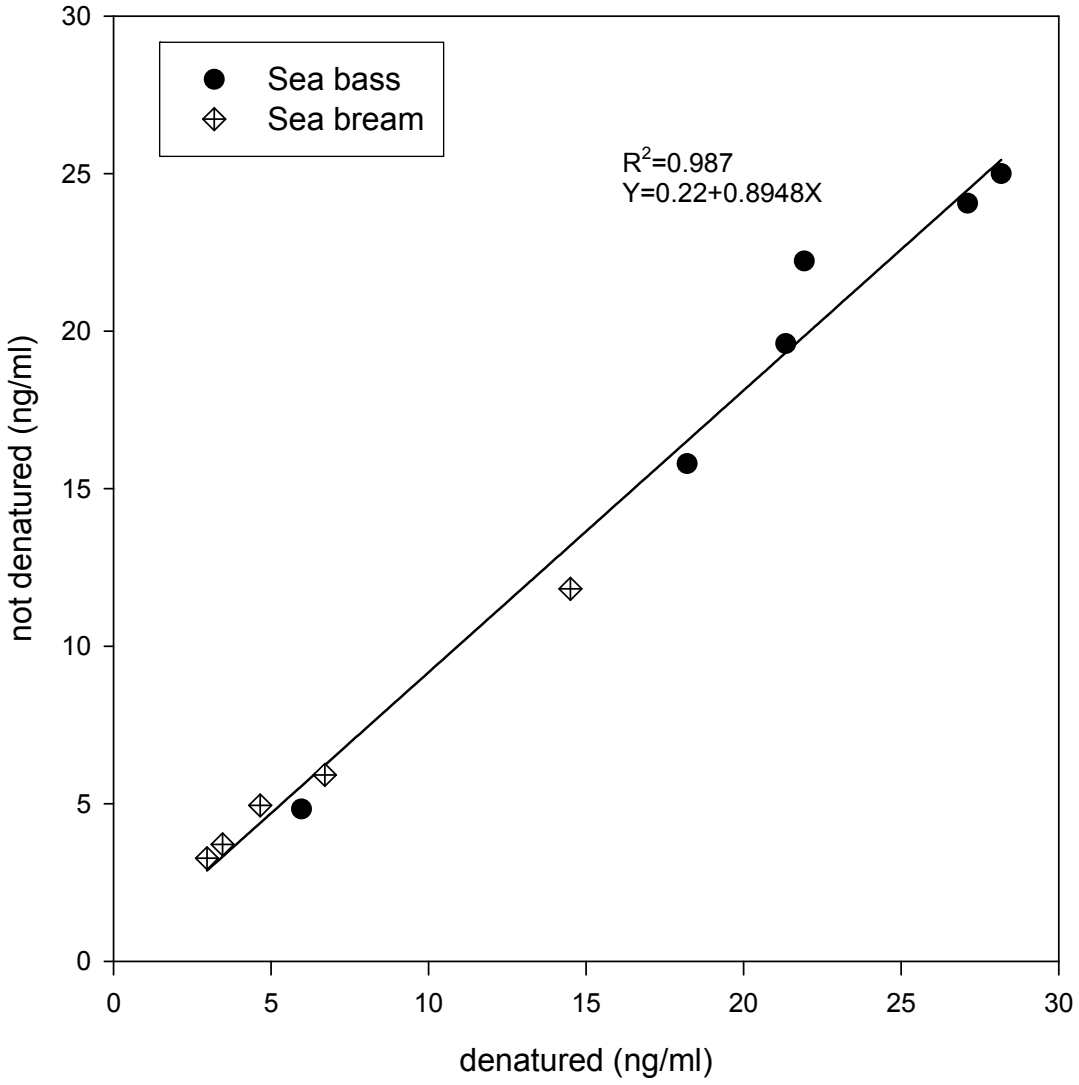


Figure 1 – Effect of denaturing plasma samples on plasma cortisol levels in sea bream and sea bass. Plasma samples were diluted in assay buffer and either denatured for 1 h at 80ºC or assayed directly (not denatured).

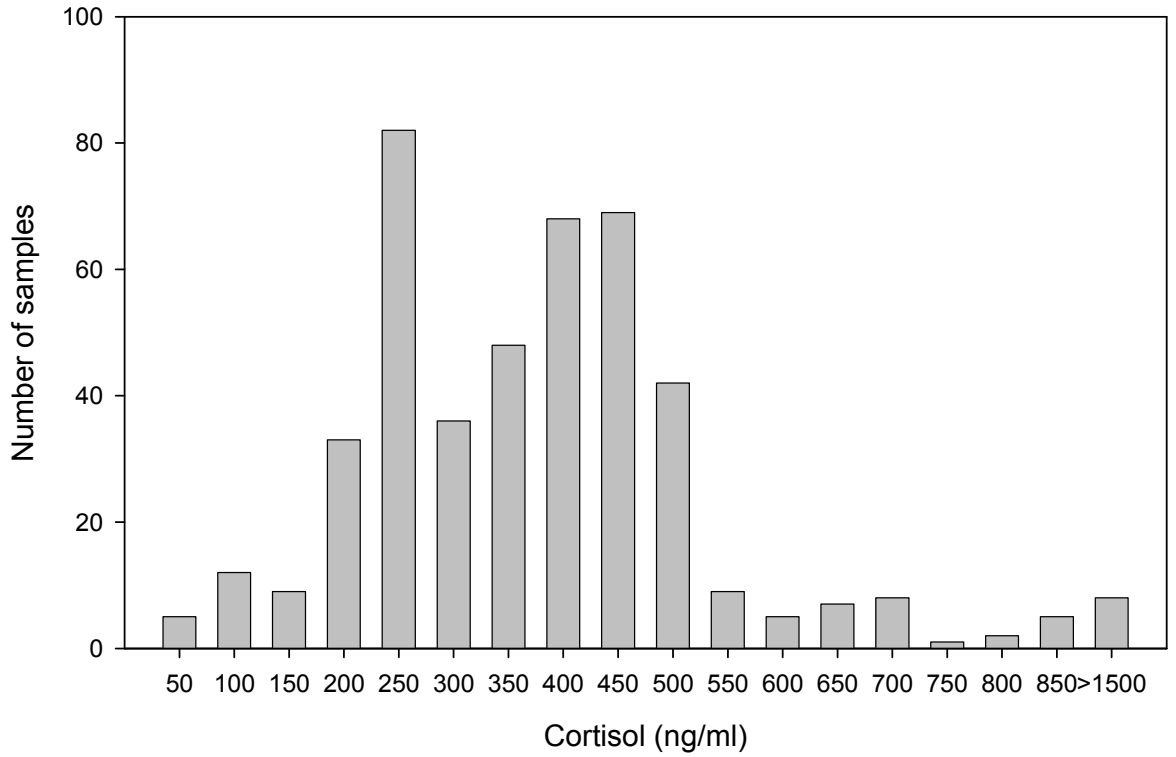


Figure 2 – Frequency distribution of plasma cortisol levels in 25% of sea bass samples from QTL stress experiment. Each class number refers to levels smaller or equal than the indicated.

3) Choice of markers for whole genome QTL scan in sea bass

a) *Marker choice* - Markers for the QTL scan have been chosen based on the latest linkage map available (December 2005). The markers cover a total sex-average length of 1506 cM corresponding to 120 markers each separated on average 13 cM. Priority was given to EST based loci and number of polymorphisms per locus (loci with highest number of alleles). Finally, 116 markers from first 25 linkage gropus were chosen.

b) Multiplex development – In order to speed up the process of genotyping and to make it cheaper multiplex PCR has been developed taking into account compatible loci based on primer sequence and known allele range. After optimization 8 multiplex reactions were developed with respectively 10, 12, 9, 12, 11, 7, 8 and 9 loci (Table 1). An eighth multiplex of 12 loci was also developed with markers recently added to the sea bass linkage map data; although a loci have not been assigned yet (unknown linkage position) (Table 1).

c) Samples already genotyped - 56 individual samples of the progenitor population were purified and genotyped for 69 loci (7 multiplex). Of 2122 individuals of the progeny (experimental population), 868 blood samples were purified and genotyped for 31 loci. Genotyping was carried out with Genemapper v.3.7 and calculation of of He, Ho, #alleles, PIC with Cervus v.2.0. Progeny assignment was carried out with Cervus and Papa software (allocation/ sexed) (Table 2).

d) Remarks: locus DLA0275e monomorphic; locus DLA0166: weak reaction and sometimes nonspecific; Loci DLA0021, DLA0038, DLA0164, DLA0166, DLA251e, DLA0254PXN had excess of homozygotes; Loci DLA0026, DLA0032, DLA0036, DLA0039, DLA0040 and DLA0164 have alleles in progeny (low frequency) not present in the parents (added individuals?).

Table 1 – Loci in each multiplex reaction

Multi01	**Linkage Group**
DLA0275e	6
DLA0051	1
DLA0049	7
DLA0021	1
DLA0162	1
DLA0164	1
DLA0122	1
DLA0038	1
DLA0016	1
DLA0233e	8

Multi02	**Linkage Group**
DLA0237PY	1
DLA0167	1
DLA0149	5
DLA0009	7
DLA0166	4
DLA0273e	10
DLA0200	2
DLA0251e	1
DLA0104	2
DLA0118	2
DLA0036	2
DLA0106	2

Multi03	**Linkage Group**
DLA0254PXN1	16
DLA0131	2
DLA0032	5
DLA0039	2
DLA0040	14
DLA0133	3
DLA0267e	11
DLA0272e	6
DLA0026	3
Multi04	**Linkage Group**
DLA0047	17
DLA0238CY	6
DLA0256e	10
DLA0121	9
DLA0008	24
DLA0146	10
DLA0117	4

DLA0261ER	17
DLA0223e	14
SaGT41b	3
DLA0134	1
DLA0175	4
Multi05	**Linkage Group**
DLA0193	10
DLA0234e	25
DLA0227e	8
DLA0135	11
DLA0257e	14
DLA0192	8
DLA0035	14
DLA0277e	15
DLA0142	15
DLA0159	19
DLA0280e	8
Multi06	**Linkage Group**
DLA0148	4
DLA0128	7
DLA0258e	4
DLA0248	15
DLA0230e	6
DLA0203	20
DLA0170	7
Multi07	**Linkage Group**
DLA0274e	5
labrax-8	16
DLA0252e	17
labrax-13	5
DLA0119	14
DLA0141	12
DLA0231e	5
DLA0216	6
Multi08	Linkage Group
DLA0060	Unknown
DLA0061	Unknown
DLA0064	Unknown
DLA0066	Unknown
DLA0068	Unknown
DLA0070	Unknown
DLA0073	Unknown
DLA0075	Unknown
DLA0078	Unknown
DLA0080	Unknown
DLA0081	Unknown
DLA0086	Unknown
DLA0089	Unknown

Table 2 - Assignment results from 868 offspring with 31 loci.

Cervus software, 5 offspring not allocated:
- P02 x P37: 65
- P21 x P33: 77
- P28 x P37: 246
- P44 x P37: 151
- P48 x P33: 70
- P56 x P33: 45
Papa software (allocation / sexed), 5 offspring not allocated:
- P02 x P37: 70
- P21 x P33: 89
- P28 x P37: 250
- P44 x P37: 153
- P48 x P33: 91
- P56 x P33: 83
Comparing both softwares: 750 individuals from 868 have common parents:
- P02 x P37: 65
- P21 x P33: 76
- P28 x P37: 246
- P44 x P37: 151
- P48 x P33: 69
- P56 x P33: 45

4) Choice of markers for parentage assignment and whole genome QTL scan in sea bream

Due to the late arrival of sea bream samples, DNA extraction was performed for only a few specimens: all breeders (177 fish) and 384 (4x96) individuals of the progeny. These samples have already been genotyped for multiplexes 01 & 02 (10 loci in total, see Table 3) but thus far assignment tests have not finished. DNA extraction and multiplex PCRs are also going to be pursued with the rest of the experimental population (2200 fish). In case that the two multiplexes are not sufficient to correctly assign progeny individuals to their parents in the breeders, a third multiplex was optimized to be promptly used.

Table 3 - Multiplexed loci developed in sea bream [Multiplex-03 was kindly provided by Dr. Luca Bargelloni (UniPD) and slightly modified at HCMR].

Multi-01	Linkage Group
Sai-12	15
Sai-14	15
Sai-19	1 (unordered)
Sau-G46	Unknown
Simbc-26	24
Multi-02	Linkage Group
Simbc-F6	13
Simbc-F7b	11
Simbc-25	14
Sau-I47	17
Sau-E82	Unknown
Multi-03	Linkage Group
EID-05	7
DD-16	25
BD-61	22
BD-86	3
DD-41	17
DD-56	18
ED-01	14
CID-03	13

Choice of markers to be used for QTL scan and multiplex development – A first-generation genetic linkage map for *S. aurata*, based on 204 microsatellite markers was recently published, and twenty-six linkage groups (LGs) were identified with a total map length of 1241.9 cM (Franch et al., 2006). However, one of the objectives of another workpackage of the present project (WP10) is to further enrich this linkage map and to evolve it from medium to high resolution map not only with microsatellites but also with genes (including some involved in stress response and disease resistance/susceptibility). Nevertheless, the sea bream linkage map presently contains very few genes and/or ESTs and effort has been put toward this direction. As also for the sea bass QTL analysis, priority will be given to EST based loci and to loci with high number of alleles.
A second generation linkage map is planned to be constructed when no less than 60-70 additional loci will be available and this is planned for March-April 2007. The design and optimization, however, of multiplex PCRs has already started and will be applied as soon as the first families will be evidenced by the parentage assignment analyses.

5) development of SNPs for candidate genes for stress in sea bass

- Data description

The European Network of Excellence Marine Genomics Europe developed about 30,000 ESTs from 5 F1 offspring from wild Atlantic parents. Fourteen normalised cDNA libraries, corresponding to 14 distinct tissues, have been developed.
A total of 33,904 ESTs, of which 29,260 are of good quality, have been sequenced at the Max Planck Institute for Molecular Genetics (MPI-MG). These 29,260 ESTs have been processed in an EST pipeline at the MPI-MB. Of the 29,260 processed sequences, 55.1% (16,117 ESTs) were redundant and thus clustered; 44.9% (13,143 ESTs) remained single.

The ESTs developed using stressed fish have now been added to the previously described dataset but not yet incorporated in this study.

- In silico SNP detection

To avoid the detection of sequencing errors, a redundancy of 2 is required for a mismatch to be considered as a candidate SNP. Thus, only contigs containing more than 4 overlapping sequences can be analysed. These contigs were selected. As shown in the following table, 975 clusters can be studied. However, the last contig contains too many sequences: its analysis may lead to the detection of false-positive candidate SNPs. It is thus dropped from the analysis.
974 (21.3%) contigs qualify for in silico SNPs discovery, representing 5548 (19%) ESTs and 477,224 overlapping base pairs.
Various tools are used to detect candidate SNPs. So far, 3 tools were tried (SNPServer, PolyBayes and QualitySNP) on the described data set; others, such as PolyFreq and MiraEST will be used in the near future.

1. SNPServer

SNPServer (http://hornbill.cspp.latrobe.edu.au/snpdiscovery.html) is an online tool developed by PBC Bioinformatics and using the autoSNP algorithm. This algorithm is based on redundancy only. Default parameters were used. In order to eliminate false positive SNPs candidates, discovered mismatches are considered as candidates if they appear at least (n-1)/2 times if n is odd or n/2 times if n is even, with n the number of aligned sequences at the studied locus. Two different scores are calculated to infer the probability that a candidate SNP is a real SNP: the minimum redundancy and the co-segregation score.
246 candidate SNPs (see in Table 4) were proposed by this tool, of which 56 are indels. 174 contigs contain one SNP or more, thus 17.9% of studied contigs contain candidate SNPs.

Table 4 - Description of SNPs detected by SNPServer

Total Number of SNPs	**246**
Mutations	**190**
Number of transitions	121
Number of Transversions	69
Indels	**56**

SNPServer detects 1 SNP every 1940 base pairs, or 1 SNP every 3.96 contigs, or 1 SNP every 22.55 ESTs.

2. PolyBayes

PolyBayes (http://genomeold.wustl.edu/groups/informatics/software/polybayes/) is the most renown tool used for *in silico* SNP detection. The SNP discovery is not only based on base called nucleotides and redundancy, but also on quality values, base composition of ESTs and an *a priori* polymorphic rate. For every slice, two different probabilities are calculated:
- SNP probability score, which is a Bayesian prior probability that the slice is a polymorphic site
- Variation probabilities: each possible mutation is given a posterior probability.

At first default parameters were used. 6,142 candidate SNPs were detected. Since the aim of using different SNP discovery tools is to compare their performance, only the candidate SNPs appearing at least twice in an alignment of a minimum of 4 ESTs are studied. The selection of these candidate SNPs reduced the number of detected SNPs to 772, described in Table 5. Some contigs of around 1,000 bp contain more than 10 SNPs, that is to say 1 SNP every 100 bp. Other parameters were then tested to reduce this

result. However they did not have a major influence on these particular contigs. Further studies of the data set showed that many indels are detected by PolyBayes. Indeed, out of the 6,142 candidate SNPs proposed by PolyBayes, 1,015 (16.5%) are indels. Out of the 772 selected SNPs, 231 (29.9%) are indels. This big number of indels is partly due to the fact that a 3bp indel is considered as 3 different indels.

Table 5 - Description of SNPs detected and selected by PolyBayes

Total Number of SNPs	**772**
Mutations	**541**
Number of transitions	367
Number of Transversions	174
Indels	**231**

PolyBayes detects 1 candidate SNP every 618 base pairs, or 1 SNP every 1.26 contigs, or 1 SNP every 7.19 ESTs.

3. QualitySNP

QualitySNP (http://www.bioinformatics.nl/tools/snpweb) is a new SNP detection tool. Its algorithm is based on a haplotype strategy to detect sequencing errors and paralogous sequences. This software can be used with or without quality values. First haplotypes are defined and classified; different filters are then applied in order to select the real candidate SNPs. Confidence scores, allele haplotype scores and SNPs quality scores are different measures calculated by Quality SNP. This tool is supposed to outperform autoSNP.
The results have not been analysed yet and are thus not reported here.

- *SNP Candidates molecular validation*

The molecular validation of these candidates requires the development of primers. Primer3 is used to design primers surrounding every candidate SNPs. The primers have to be at least 25 bp removed from the SNP and the product length has to range between 300 and 400 bp. The design of such primers limits the number of SNPs that can be checked. Some candidate SNPs cannot be validated for the following reasons:
the position within the consensus sequence is too close from the sequence end
no primer pair has been found

Table 6 - Primer development results

Number of primer pairs	**SNPServer**	**PolyBayes**
Successfully designed	218	699
Non successfully designed	28	73
Too close from the end	14	28
No primer found	14	45

Consequently 218 (88.6%) SNPs detected by SNPServer and 699 (90.5%) SNPs detected by PolyBayes may be validated (Table 6). Out of these successfully designed primer pairs, 41 SNPServer candidates and 144 PolyBayes are located too close from the consensus sequence end; they may be tested later. Both common primers and SNPServer primers were optimised, results are presented in Table 7.

Table 7 - Primer Optimisation

	Number of primer pairs
Ordered	118
Successfully optimised	61
Non successfully optimised	57
- No amplified fragment	26
- Aspecific reactions	29
- Too weak reactions	2

At first, heteroduplex analysis was used to validate candidate SNPs. However this technique is no longer used for different reasons: heteroduplex analysis results cannot be unambiguously analysed, almost all contigs contain one or more SNPs and sequencing is required as a last validation step. Instead, 8 wild European sea bass individuals are sequenced. The individuals chosen for sequencing originate from 2 populations: 4 originate from the Mediterranean Sea and 4 from the Atlantic Ocean. The Atlantic Ocean population is composed of 3 individuals from North Sea and one pooled DNA sample containing the DNA of the 5 individuals used to produce the cDNA libraries.
So far 47 primer pairs (out of the 61 successfully optimised) were used. In total, 127 SNPs and 5 indels were detected in 40 loci. 85.1% of the selected loci contain SNPs. Two loci were mitochondrial DNA. While sequencing genomic DNA, new sequences were discovered. Theses sequences must be introns and are polymorphic: 42 SNPs were found in 20 introns, representing 1 SNP every 113.31 bp. 85 SNPs were discovered within ESTs representing 1 SNP every 123.21 bp. The number of SNPs in sea bass genome is thus on average 1 SNP every 119.91 bp.

Figure 3 represents the number of SNPs detected by sequencing per locus.

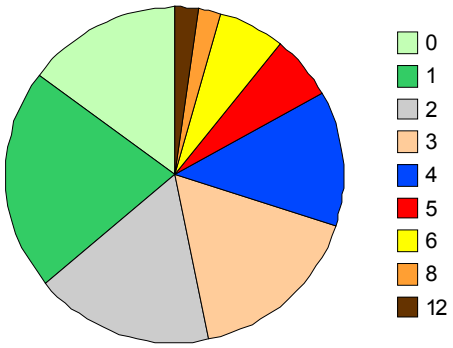


Figure 3 - Number of SNPs per locus

- Comparison of SNPServer and PolyBayes SNP candidates

173 SNP candidates were detected by both tools, representing 70.3% of the SNPs detected by SNPServer and only 22.4% of the SNPs detected by PolyBayes.
Table 5 and figure 2 allow the comparison of the different kinds of SNPs detected by different methods.

Table 8 - Description of SNPs detected by different methods

	Number of transversions	***Number of transitions***	***Number of indels***
SNPServer	121	69	56
PolyBayes	367	174	231
Sequencing	89	38	5

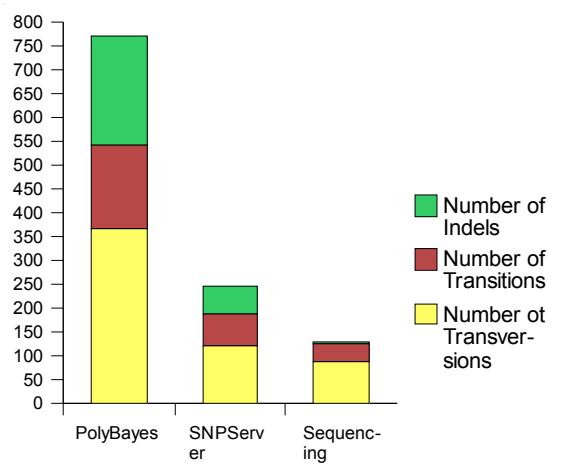


Figure 4 - Description of SNPs detected by different methods

Among the 85 SNPs detected in the ESTs, 20 (23.5%) were detected by SNPServer and 21 (24.7%) by PolyBayes (Table 9). 17 SNPs were detected by both tools. It is thus useful to use both tools. Some of the detected SNPs were not present in the panel used to make the cDNA libraries and thus could not have been detected bioinformatically. The pooled DNA will be cloned in order to estimate the real number of SNPs that were present in the initial panel. This will allow the calculation of a real success rate of *in silico* methods.
The SNP detection tools also detect candidate SNPs that reveal not to be SNPs. SNPServer and PolyBayes detected respectively 18 and 13 such candidates (Table 9). 10 false positive SNPs were detected by both tools. The fact that SNPServer detects more false negative than PolyBayes may be due to the fact that no primer pair was ordered for candidate SNPs only detected by PolyBayes.

Table 9 - Validation of SNP discovery tools

	SNPServer	***PolyBayes***	***Both***
Number of true positive	20	21	17
Number of false positive	18	13	10

- Incorporation of stress genes ESTs and BAC end sequences

New ESTs have been added to the initial dataset. The contigs containing more than 4 overlapping ESTs will be selected and compared to the one which were already studied. New contigs should appear, they will be screened for candidate SNPs using the SNP detection tools. Old contigs will be screened once more in case new candidate SNPs are

detected due to the addition of new sequences. Another tool SSAHA will also be used to add the BAC end sequences developed at MPI-MG.

Deviations from the project workprogramme

There are no major deviations from the workprogramme although delays have occurred due to two main factors: 1) The requirement to repeat the experiment with the sea bream and 2) difficulties in transporting samples in dry ice which required obtaining a specific licence.
As a result deliverable 7.03 has been delayed from month 18 to month 27 and deliverable 7.04 has been delayed to month 32. The delays do not

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D07.01	Sampling 700 individuals progenies of seabream for confinement stress response **(updated)**	Month 12	Month 24
D07.03	Blood parameters measured for all specimens	Month 18	Month 27
D07.04	Development of SNPs for candidate genes for stress; genotyping of the grandparents, the parents and about 500 progeny of the seabream and seabass populations	Month 24	Month 32

List of milestones

Code	title	Due date	Actual/foreseen achievement date
M10	QTLs data contributing to the phenotypic variance for stress in seabass and seabream.	Month 42	Month 48

WP08: Radiation Hybrid (RH) panel construction for seabass

Start date or starting event	1
Activity type [1]	RTD/Innovation
Participants	CR16
Person Months	22

Objectives

1) To produce a panel of hybrid cell lines resulting from the fusion of seabass cells and hamster cells that would allow within WP09 the mapping of a minimum of 1500 markers

Progress towards objectives / deviations from the project workprogram

Although many RH panels have been constructed with different mammalian cells as donor cells, very few have been constructed with fish cells due to many technical difficulties encountered at different stage of the construction process. So far only one sea bream panel was constructed using freshly made fibroblasts. Two zebra fish panels have also been constructed but with a zebra fish cell line as starting materials.
Because of the close proximity at the phylogeny level of sea bream and sea bass we anticipated that the method we developed for the construction of the sea bream panel would be readily adaptable to the construction of the sea bass panel. However as reported last year we encountered many difficulties.
Preparation of large number of fibroblasts poses a problem.
Far less cells diffused from fine explants
Released fibroblasts grow poorly and do not rapidly accumulate to a minimum of 20.1O6 cells which are needed for a single fusion experiment
Sea bass fibroblasts appeared more fragile than sea bream fibroblasts
to γ irradiation - a larger number of cells being destroyed – often resulting in a very low number of cells to perform a fusion
to trypsin treatment
The rare cloned we have obtained grew poorly and were lost.

We had also to face a problem of massive yeast contamination, most probably due to the fish regimen that contains yeast extract.

These were the main conclusions we reported at the meeting hold a year (November 2005) ago by the whole consortium in Las Palmas, Gran Canaria.

This year was then devoted to the testing of several cell growth conditions as well as cell sources and as we reported at the Heraklion meeting (November 13-14, 2006) we believe all the problems are now solved.
Essentially, we were able to produce enough cells from fresh spleens prepared from a new source of fishes. In short, spleens were dilacerated by gentle pippeting, irradiated at 3000 rads and fused to Hamster HPRT$^-$ cells. Fifteen days later, small colonies started to appear. Well individualized colonies were recovered and further cultivated in small plastic bootles (25cm^2). Three weeks later, cells were recovered and DNA extracted. Following this new strategy, we have been able to make 3 fusions and harvest 360 independent clones. DNA has been extracted from 140 clones and further WGA amplified. And we have recently cstarted to analyze the sea bass DNA content of a number of clones. For that we selected 108 markers well distributed on the meiotic map and tested their ability to be amplified on sea bass DNA and give a well defined DNA band and not on hamster DNA; 96 markers passed this test. We then checked the retention of these markers in 44 hybrids.

From this experiment we could estimate that (1) all hybrids retained some sea bass DNA (between 4.4 and 51% of the markers were retained by this sub set of hybrids) (2) the average retention of the panel would be 25.4% (3) given these numbers, we strongly believe we are in a good position to select out of the 360 hybrid clones a panel of 93 hybrids that should have a good retention value and provide a good representation of the sea bass genome.

List of deliverables

None the first year but the following will consist in a series of 92-94 hybrid cell lines (Seabass/hamster) that will be selected among many more. Selection will be based upon the percentage of seabass DNA present in each hybrid.

List of milestones

We anticipate the WP8 should terminate in spring 2007

WP09: High resolution mapping of the sea bass genome based on the bASSMAP map

Start date or starting event		1	
Activity type [1]		RTD/Innovation	
Participants	CR12	CR13	CR16
Person Months	10.1	12	31.49

Objectives

To produce a medium-resolution ordered sea bass linkage and gene map containing uniformly distributed type I and type II markers
A comparative genomic analysis with other fish and vertebrate maps.

Progress towards objectives

Developing and mapping microsatellite markers and SNPs on the seabass linkage map:
Microsatellite markers are isolated and characterized from ESTs and candidate genes obtained in this project (see WP03) as well as from public sources and agreements with ongoing projects (eg EU NoE Marine Genomics Europe and MPI-Molecular Genetics project on BAC end sequencing). New microsatellites are tested for variability and reliability on 20 outbred wild sea bass. The genotypes of these microsatellites are scored on a well-characterized panel of sea bass including 2 parents and 50 progeny (Venezia Fbis) for mapping purposes.
Partner 12 – KULeuven: About 250 new liver ESTs deposited to GenBank in April 2005 have been searched for microsatellites. Ten primer pairs have been designed; 4 of those showed strong homology with known genes and are valuated. Three of these 10 were polymorphic for the Venezia Fbis family (DLA0282e till DLA0284e) and have been submitted to the ResSpecies database (http://www.resspecies.org/).
Marine Genomics Europe (http://www.marine-genomics-europe.org) developed about 30,000 ESTs from 5 individuals from Atlantic wild parents. Fourteen normalised cDNA libraries, corresponding to 14 tissues, have been developed. After aligning and assembling all sequences, they were screened for repeats. 953 clones were found containing repeats. This cDNA library was used to search for microsatellites with special attention to tri repeats. From these 94 primer pairs were developed and 49 showed polymorphism for the Venezia panel (DLA0285e till DLA0333e). Forty three showed polymorphism in the Venezia Fbis family and are ready for mapping. All those markers and genotypes have been submitted to the ResSpecies database (See also Annex 1).
SNPs were developed from the same 30,000 ESTs produced by Marine Genomics Europe (see WP07).

Partner 13 – HCMR: Twelve new polymorphic microsatellites markers from a BassMap enriched genomic library were produced at HCMR (DLA0090-DLA0100). Furthermore, gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) MGE-ESTs datasets were screened for di-, tri-, tetra-, penta and hexanucleotide repeated motifs. According to the search criteria adopted, 1604 ESTs that harbor 1748 simple sequence repeats (SSRs) were identified in both species. We used as template the non-redundant EST-SSRs; for sea bream and sea bass, 91.32% (i.e. 821 out of 899) and 73.9% (i.e. 521 out of 705) of the EST-SSRs, respectively. The chance of finding a SSR-containing sequence in the non redundant EST database was thus 4.51 and 2.94%, with an average density of one microsatellite every 17.2 and 23.7 kb for sea bream and sea bass, respectively.
Primers were ordered for 260 sequences that approximately correspond to all those containing equal or greater than 15, 6, 6, 5 and 5 repeats for di-, tri-, tetra-, penta- and

hexanucleotides, respectively. When these primers were PCR checked on wild sea bream individuals but also on sea bass, we observed that 44.2% of the gilthead sea bream primers gave PCR products of expected size also in European sea bass, whereas 39.3% did not amplify.
Using a significance threshold of e<10-5, an initial *in silico* analysis (i.e. Blast searches of sea bream versus sea bass EST-SSRs) had pointed out approximately 60 sequences with good homology (~7.43%). These matches were usually due to blocks of highly conserved sequences, and between the two species they had a length of 25 to 800 bp (average 212 bp) with sequence similarities between 73 and 100% (average 85%).

Partner 16 – CNRS-Rennes:
This part consists on the mapping of a certain number of markers onto a radiation hybrid panel that is under construction (WP08). Due to several technical difficulties this panel is not ready yet, although all difficulties encountered have been solved and 350 hybrid clones recovered (see WP08 report). The selection of the most suitable hybrids is in progress and should last a couple of months. It is anticipated that the typing of markers on this panel will start in April/May next year.

Database maintenance:
Entry of marker genotype data continues to the ResSpecies database via the web, with partners 12 and 13 submitting the data as they are generated in the laboratory. Data are screened upon entry and those containing Mendelian errors are flagged and rejected by the database. This allows submitting scientists to rapidly identify errors in the data and correct these before final submission of the data. The database accepts microsatellite, AFLP and EST data, and has a commercial database management system. It harbours >200 AFLP markers, 300 microsatellite markers and 20 ESTs available from the previous EU project BASSMAP (as of 31.10.05). Tools are put in place to export the data in several formats, including the appropriate format for linkage analysis using CRI-MAP. Access to data will be restricted to the collaborating participants for the course of the project, but may be made more widely available by mutual agreement of the project partners.

Genotyping:
Microsatellite genotyping of the panel follows a semi-automated procedure of PCR amplification, electrophoresis, scoring and database submission. Data submission is organized and quality controlled through the limited access and purpose built database ResSpecies. SNP genotyping of the panel is done through a combination of sequencing and Maldi-Tof Mass Spectrometry. Additional genes will result from ESTs developed for the functional analysis (see WP03). Partners 12 and 13 are using the Venezia Fbis European sea bass panel (2 outbred parents and 50 progeny) for mapping purposes. Partner 16 will start the mapping of the Radiation Hybrid panel next spring.

Genetic linkage map:
Markers will be built into linear maps by partner 12 using linkage analysis and the maximum likelihood software package Cri-map, which has proven to be very effective for analysis of marker data from many species including seabass. Mapping of makers on the second generation linkage map of European sea bass has been closed on 01.12.05 and a scientific paper is in preparation. The mapping of new markers (SNPs and microsatellites linked to ESTs) on the third generation map is in progress (partners 12 and 13).

Testing of seabream markers on a seabass radiation hybrid panel:
Preliminary data indicate that approximately 90 % of seabream ESTs also give a specific amplification product from sea bass. We will test all microsatellite and EST primer pairs that were successfully scored in sea bream for amplification in sea bass. Markers will be tested using the same established PCR and gel electrophoresis protocols that will later be used for radiation hybrid mapping. The use of seabream markers will rapidly populate the seabass radiation hybrid map and allow conserved synteny studies. This task will start next spring.

Radiation-hybrid gene mapping:
1500 sequence tagged sites (STS) markers corresponding to ESTs or polymorphic microsatellites will be scored on the sea bass radiation hybrid panel. The markers will be taken from the sea bream map as well as contributed by partners 12, 13 and 14.1 (and in fact the wide community of sea bass genomics). The map will be anchored by way of microsatellite markers on a genetic map of sea bass that has been produced in the BASSMAP project. The resulting data will be submitted to the project database and website. This task will start next spring.

Deviations from the project workprogram

The work programme is being followed faithfully for its content. The timing of marker development and mapping on the Radiation Hybrid panel has some delay. We will do our utmost best to have all marker submissions completed by May 2007 in order to have a linkage map by August 2007.

List of deliverables

Code	title	Due date	Actual/foreseen achievement date
D09.01	300 microsatellites and 200 ESTs of sea bass mapped on the sea bass RH panel	22	36
D09.02	primers for 300 microsatellites and 2000 ESTs of sea bream tested in sea bass	22	30
D09.03	50% of 300 microsatellites and 800 ESTs of sea bream mapped on the sea bass RH panel	26	36
D09.04	100 new microsatellites of sea bass developed and mapped	29	29
D09.05	Database Resbass stocked with microsatellite and EST genotypes.	30	30
D09.06	High density map generated	33	33
D09.07	Synteny map based on RH and linkage map.	36	36

List of milestones

Code	title	Due date	Actual/foreseen achievement date
M09.01	High quality sea bass RH map for identification of QTL genes through comparative mapping and candidate gene approach.	36	36

ANNEX 1: detailed report of microsatellite development and mapping in sea bass by partner 12 (KULeuven), partner 13 (HCMR-IMBG) and partner 16 (CNRS)

EST derived microsatellites.

About 250 new liver ESTs deposited to GenBank in April 2005 have been searched for microsatellites. Ten primer pairs have been designed; 4 of those showed strong homology with known genes and are evaluated. Three of these 10 were polymorphic for the Venezia Fbis family and have been submitted to the ResSpecies database (DLA0282e till DLA0284e).

Marine Genomics Europe developed around 30,000 ESTs from 5 individuals from Atlantic wild parents. Fourteen normalised cDNA libraries, corresponding to 14 tissues, have been developed. After aligning and assembling all sequences, they were screened for repeats. 953 clones were found containing repeats (summary see Fig 1). This cDNA library was used to search for microsatellites with special attention to tri-repeats. Out of these 94 primer pairs were developed and 49 showed already polymorphism for Venezia panel so far (DLA0285e till DLA0333e). Forty three showed polymorphism in the Venezia Fbis family and are ready for mapping. All those markers and genotypes have been submitted to the ResSpecies database and are available on request.

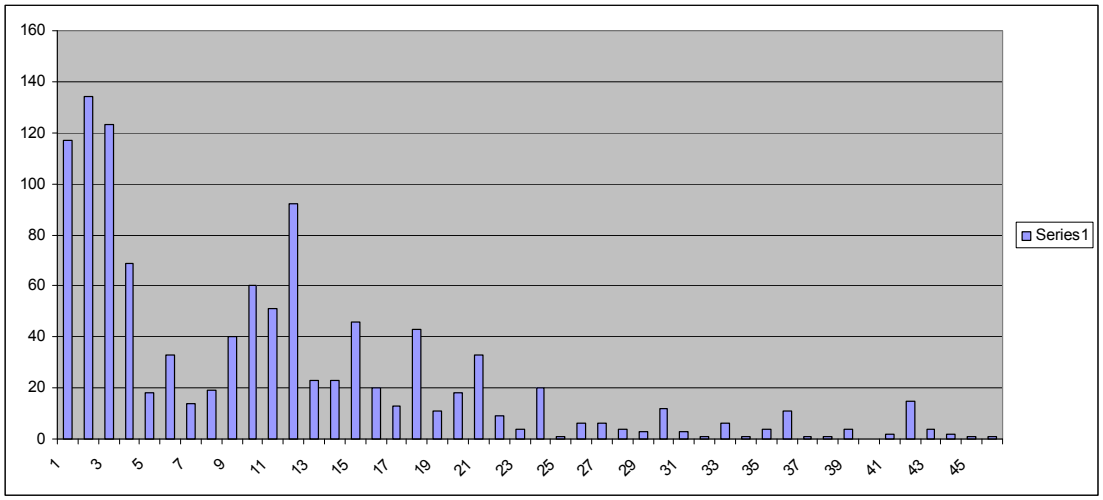


Figure 9.1: Frequency distribution of microsatellite repeats (from 1 to 47) isolated in European sea bass.

Twelve new polymorphic microsatellites markers from a BassMap enriched library were produced at HCMR (DLA0090-DLA0100). Four more markers were polymorphic in Venezia Fbis family but were uninformative on the panel. Names, PCR conditions (annealing temperature and $MgCl_2$ concentration) and characteristics (number of alleles and heterozygosities) for the above markers are shown in Table A1.1 below; for clone sequences see in annex.

Submission Numbers	Clones	T_{ann}	$MgCl_2$	Size	Number of alleles	Hexp	Hobs
DLA0090	dlz-432	58	3	154-171	6	0.6859	0.5238
DLA0091	dlz-434	55	1.5	167-173	3	0.3730	0.3810
DLA0092	dlz-460	52	1.5	243-257	7	0.7823	0.7619
DLA0093	dlz-444	55	1.5	127-157	11	0.8639	1.0000
DLA0094	dlz-457	55	1.5	185-209	8	0.6600	0.8000
DLA0095	dlz-422	55	1.5	254-300	11	0.8209	0.7143
DLA0096	dlz-416	55	1.5	256-270	5	0.7132	0.6190
DLA0097	dlz-463	55	1.5	216-240	8	0.7550	0.5000
DLA0098	dlz-420	52	1.5	164-232	17	0.9288	0.9500
DLA0099	dlz-470	58	3	163-201	10	0.8628	0.8571
DLA0100	dlz-441	55	1.5	254-338	13	0.8673	0.6190
	DLZ-442	55	1.5	237-261	5	0.3962	0.2941
	DLZ-425	55	1.5	130-144	4	0.5680	0.4286
	DLZ-415	55	1.5	210-228	5	0.6213	0.4000
	DLZ-453	58	1.5	251-257	3	0.5714	0.3810

2. High resolution mapping of sea bass genome based on the BRIDGE-MAP map

Two datasets of gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) with 18,196 sea bream unigene clusters containing 29,895 ESTs and 17,716 sea bass unigene clusters containing 29,260 ESTs, were screened to detect di-, tri- tetra-, penta and hexanucleotide repeated motifs. According to the search criteria adopted, 1604 ESTs that harbor 1748 simple sequence repeats (SSRs) were identified. We used as template the non-redundant SSR-ESTs; for sea bream and sea bass, 91.32% (i.e. 821 out of 899) and 73.9% (i.e. 521 out of 705) of the SSR-ESTs, respectively. The chance of finding an SSR-containing sequence in the non redundant EST database was thus 4.51 and 2.94%, with an average density of one microsatellite every 17.2 and 23.7 kb for sea bream and sea bass, respectively.
For the sea bream, there were finally 664 pairs of primers accepted (73.85% of the initial 899 SSR-ESTs). Primers were ordered for 260 sequences that approximately correspond to all those containing equal or greater than 15, 6, 6, 5 and 5 repeats for di-, tri, tetra-, penta- and hexanucleotides, respectively. When these primers were PCR checked on wild sea bream individuals but also on sea bass, we observed that:
-78.1% of primer pairs gave PCR products of expected size in sea bream, 8.5% gave much bigger or non-unique products, and 13.4% did not amplify at all. Preliminary genotyping analysis with approximately 20 fluorescent primers, mainly for dinucleotides, indicated that more than half of them are polymorphic.
-44.2% of the gilthead sea bream primers gave PCR products of expected size also in European sea bass, whereas 39.3% did not amplify.
Using a significance threshold of e<10-5, an initial in silico analysis (i.e. Blast searches of sea bream versus sea bass SSR-ESTs) had pointed out approximately 60 sequences with good homology (~7.43%). These matches were usually due to blocks of highly conserved sequences, and between the two species the length varied from 25 to 800 bp (average 212 bp) with sequence similarities between 73 and 100% (average 85%).

Data on 260 non-redundant EST-SSRs defined on sea bream (coded as -2- for good amplification, -1- for medium amplification or product of much different size (>< 50bp) and -0- for no product).

In sea bream:
203 have a PCR band of expected size (78.08%)

22 give a product of different size (8.46%)
35 give no product (13.46%)

The same 260 EST-SSRs defined on sea bream, gave the following results in sea bass:
115 have a PCR band of expected size (44.23%)
43 give a product of different size (16.54%)
102 give no product (39.23%)

106 good both in bass and bream (2-2)
10 medium in both (1-1)
29 no product in both (0-0)
30 (2-1)
67 (2-0)
6 (1-0)
6 (1-2)
3 (0-1)
3 (0-2)

From those 106 good both in sea bass and sea bream (2-2), 29 are annotated in the MGE pipeline.
31 are di-nucleotides (8 annotated)
51 are tri-nucleotides (15 annotated)
18 are tetra- nucleotides (5 annotated)
3 penta-nucleotides (no annotated)
3 hexa- nucleotides (1 annotated)

Annex 2:

i) Sequences of SSRs mapped on Venezia Fbis family and panel:

>DLA0090_dlz-432
CCACATTAGCAGAAGTAGTGATGACGGCAGCAGGCATTCAACAAAAGATTGAGCAGGCCA
CATGTGTTAAAACACACAGCCATTATGGAGACAGTGTGTAGGATGCAAGTAGTTGTGAGC
TTGCAGGGTTTGCTTTAGAAACACACACAGACAGACAGACACACACACACACACACAC
GCACACACAGATTCAATGAGAGTCTAGCCAAATAAATGCGGCAGATGCTATTAGCACCCA
NGTTTCAGT
>DLA0091_dlz-434
ACGGGTTTCGTGCGAACTTCCCAGGGTCGACTGGGTTAGTCTGCTACACTGCAGTGGAAT
CACTGGACTTANGCTGCCTCCTGCGGTGGGATAGTCCCAATGCCAGTCCTAATACCACCT
ATACCGTGCAGACAAAGACTCANGGGTAAGTCCTCCTGTCTGTGTCTGAGTGTGTGTGTG
TGTGTGTGTGTGCATGTGCTGTAACTTCACACATTTAGTTTTAGCTGCTACAAGGACAAT
ATCTGGAATTATCCGCACACGCAAGAGATTTAATGCTGTCAC
>DLA0092_dlz-460
CTGGTATCAGAGGCTTAGATGTGCAAAGTTGCTCTTTTAACTTTGGAGTAGCTGGTGAGT
CAGTGTCTGTGTCTATATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTTGCAGCA
GTATGATGAAAATTCTTGGAGCATAAAGGCNCTTTTATCATCCCCAGAATGAGAGACTGT
CACTGTCTGGTTAGTGTGCGTTACTATGTGCATGTGTGTATGCAGCCAGAGGTAGAAAGC
GAGAGGTAAGCGCAGCCTGGT
>DLA0093_dlz-444
CTGACTAAATCACATAACATCTCTTANATCGTAAGGTGAACTATAACGAGATAGATTTTA
ACATCATTCCAGTAACAACAGATTAGTAGAAAGAGGGAGTAGTTGGATGGTATTTTAATA
GGAGGATTTATTAATTTTCAGTGGATTCATATTGTGGTGATTTGAACATATCTGCATATA
ACTCTACAGTCTAAAGAAGAAACTTTATTCCTTAAGTTANTTGATTTACATATAAAGCTT
GATTCGATGTAATGTATAACAGTGTAAAATTTTGACATATCANNGAACAGTAAATATCAA
TAGGAGCAATTGTTTTCATATATGTTTATAATGCANGCTTTTACCCAGCATTCACAGTGT
TGTGTCCTTGAAACTGACCCTGTTGAGTGTTTTACAGAAGAGAGCAGCTGTGTGTGTGTG
TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCAAGGGCTAACTGTCTTC
TGCGACTGAAGCATTCATTAGTTCACTGGTCAGGTGGAAATGTTTTATTCAGGGGGTCT
>DLA0094_dlz-457
ACGGCTGGGGAATGCTGAGATTATGACAAGAATAGCCCAGCCATTTGAAAACGGAAGGGT
TCTGTTGTTTACGTTGCCAGAAATGTGTGCTTACACAGCCTTTAAGTAGGCTGCAGAGCT
CCAAGGCCTCCAACAGGGTCTGGTTCCACTTGTTACATCCAGACTGGAGTATCTGGNGTT
CGTGCTGTTCCAGCTGTCCAGAAATATGCCTGCGTGTGTGCGTGTGTGCGTGTGTGCGTG
TGTGTGTGTGTGTGTGTGTGTGTGTGCATCTATACACTGGAACTGTTGTGTTCATAACCT
GT

>DLA0095_dlz-422
TGTGTGAGCCAGCTGAAGCTCCTCCAGTCCGTGTTTGCCCAGGTGATATCTGGCCAAGTC
TTTCCTGGTCACCAGTCCAATCACCTGCAGAGCATCACAAACCAAAAAATGTGACGTCAG
AACAATCAACGTTGTCATCAATATCATTATCATCATCATCATCATGGCATTCAGTAT
CAAGAACATGATTATTAACCATAACTTTAATCAATGCATANAGATGCCAAAATACACAGG
GACATAAAAAGTAGATAAAGGATTGACACAGANACACANACACACACAATTCAATTCAAT
TCAAAGAGGCTTAATTTGCATCAGAGACATTTTCATTTCAAAAAGGTATATGTATATACA
GATTAGTGAAAAAATAACTATATATGAACCCACTTTTCCCTTTTTTAATGAATGTATTTG
TGTGTGTGTGTGTGCGCGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTA
TTTGTGTGTGTATTTGTGTGTGTGTCTGGGTCTTATACTCTTTACCTGTTCTGATCATCC
ACTACCACCAGTGTCTGAGTCCCAGCGCTCTGAACAGCTTAAACACACGAGGCAGAGACG
TTTCCTGCAGACAAACCGCCTCATTTTACTCCCATCAA
>DLA0096_dlz-416
AACTTAGTGAAGTAACTTGTGGCAAAATGTTAAATCTACCAACCTATTTCAGTGTGTGTG
TGTGTGTGTGTGTGTGTGTGTGTGCAGTTGCCTGAGTAGAACATGCTTTATGTGGACGAC
GAAATGGAAGGACATTCTCTAAACATGCTTTTCCAAGCTTCAGTCACTATAATATGAGTG
TATGTGTATCANACAGTGAACCCCACCCAAATATGACTTGNGAGGTCCATACAGTTTAGA
GGGTCCTGTCCTAGATGCATCGATTTGGTTTTTAAGAAAACTTGGACTGCCTGCATTTGA
TCTGTATGTTAGTTTAATGTGAATGGTGAATTGTTTAGGT
>DLA0097_dlz-463
CTGAGCTGGTAGAGAGCAGCAGAGCACGTATAAATAGCCGTGAAGGAAGGGAAGGACTGG
GCTGCTGCAGGAGTGTGAGAGGGGGTTAATCACCAGCTCATTAATTAATAACGAAGATAA
TTAGCTGAGTGTGAAGACGAGTTCTGTGTGTGTGCGTGTGCGTGTGTGTGTGTGTGTG
TGTGTGTGCTGCAGTGGGGGCAAAAAACTGCAATTAGAAAAATGTGTTTATCAGTGAATT
CTGCTAATAACCATACTGGAGTCGGTTTCTTCCTCGAGTCTCTCGCCGTTCAGTAAAGAT
GCAGCTGGACTGTTAATATTTGATAT
>DLA0098_dlz-420
CTGTGACCGCGGGACGGACCGAAACACAGAAAACGGTATCACAACAAGAACAACAACAAC
AGCAGCAGCAGGCTGATAATCTGCTGCACCTGTTCCTCCTCCACCCTGCACACACATTTC
ATAGCACACTGAATATCAGACCATGATCACAATCACACANAGGGAAGCTCCTCTGGGTGA
CTGAGTCACCGCAACTCACGCATCTGTTACATCATACCCGCATTATTTATTCAAACGCGG
TTAAAACCCGTAAATCTGATGTGAATAAGCTGCANGATCTCACACACACACACACACACA
CACACACACACACACATATATATAAACACANGTCCCATAATGAGGATGAAGCCACATTAC
TGTGCTGGAGCTCATACAGCTGCTCACACAGCTACAGGACAGTAAACTGTATTAATTGTG
TG
>DLA0099_dlz-470
CACATTGGGTAAGAGGTAGACTCTGGATAGGTCACATGTCTAGGCTAAGAAATATAGACA
GACAACCACATTTACACACACACACACACACACACACACACACACACACACACACACACA
CACACACACAGGTTTAAAAAAAAAATAATTTTAGTAGCTGCTCTGAGAGTTTTGACTGTAT
AATACCGTCCTCATCCTCTTTATCATCCATTAGACAAACTCCACTCTGTCATGTTGNTTT
TCAGGTCAATAATGATGGTTTCATGACATTAACAATTTTCATCTTANAAACAAAGGTCAA
GT
>DLA0100_dlz-441
ACTTTTTACATCCAAAGCTCCATGCTGATATGGAACATCAAAGACTGGCATAACCATTAC
CAGGCAGTTTTCGCTCACTGTGGCAATAATAATGTCCATGATACTACAAACCATTTTACT
GTGGATGGTAATATAACAGTGAATCACAGTGTTGCGAATTACACATTANGCTGANACAAG
GCTCCACAAATGAACCCCATTAGCATGGAGCCGACTAGTGTGAATTAGTGTGTGTGTGTG
TGTGTGTGTGTGTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGATTGTTTGGGTGCCATTT
AAGAACAAGTCATGCAACGCTGACATANACAAACAACATGAATCAACATACACACACACG
CGCGCACCTCTGGTATCTGTTCTGTTGATACTTACATAATTGTCTCGAGCAGACCAGCCT
GGGTAGAGCTGCATGTGGAGCTGCCGTTCCNTGCGGGCTAACTCATAATACTTAGCTTGC
TCTTCCCGAGATAAAGCATGGCACTGTTTCAG

ii) Primer sequences of SSRs mapped on Venezia Fbis family and panel:

DLA0090_dlz-432
Forward GAGCAGGCCACATGTGTTA
Reverse TGGCTAGACTCTCATTGAATCTG

DLA0091_dlz-434
Forward CCAATGCCAGTCCTAATACCA
Reverse TGCGTGTGCGGATAATTC

DLA0092_dlz-460
Forward TCAGAGGCTTAGATGTGCAA
Reverse AGGCTGCGCTTACCTCTC

DLA0093_dlz-444
Forward AGCATTCACAGTGTTGTGTCCT
Reverse AGTCGCAGAAGACAGTTAGCC

DLA0094_dlz-457
Forward TAGGCTGCAGAGCTCCAAG
Reverse AGGTTATGAACACAACAGTTCCA

DLA0095_dlz-422
Forward CAAAGAGGCTTAATTTGCATCA
Reverse GAGCGCTGGGACTCAGAC

DLA0096_dlz-416
Forward AACTTAGTGAAGTAACTTGTGGCAA
Reverse TCGATGCATCTAGGACAGGA

DLA0097_dlz-463
Forward GCTGCAGGAGTGTGAGAGG
Reverse GCGAGAGACTCGAGGAAGA

DLA0098_dlz-420
Forward CGCAACTCACGCATCTGT
Reverse TGGCTTCATCCTCATTATGG

DLA0099_dlz-470
Forward GAGGTAGACTCTGGATAGGTCAC
Reverse AAGAGGATGAGGACGGTATTA

DLA0100_dlz-441
Forward CTGGCATAACCATTACCAGG
Reverse GCGTTGCATGACTTGTTCTT

iii) Sequences of SSRs found polymorphic and genotyped on Venezia Fbis family but were not informative for the mapping panel:

>DLZ-442
ACCTGGCCAATAAAAAACCCCATGTGACAACAATACAATTTAGAAATGGTGCAGGGTTGA
GGAAAACACCACTTGTTAATCCTGGAGAGTTATAGATTATAGATTCAGGGAAGAAAAACG
ACGACAGCATGATGAGTTATGATGTTCATCTCATGGGGACTTTGTGGGCCTGGTGGCTTG
AGGCCCTTCGGCAACCTTGTTGCGTGTGTGGATGCACACAATACAAGCACACACACACAC
ACACACACACACACACACACGTGGGGGCCACCTGCCACACACACAAAGATCCTTACTCGG
TTATTTGCTTTGTGCAGTGAGTTATCCATGTGTTGGCATGCTCTGTGGCCATTAACTGAT
GTGCTTGGCTACATAGACAGAAATGATTTCTGGT
>DLZ-425
ACACTTAGTGCTCGACTTCGACTATGGTCAGTTTGAAGGCACTGTAAGAGGTGTAGCCAC
CTCCCAGCTTACAGGCTGTGCTATGACCTTTAATTCAATGCCAAGGAGAGCATAAACACC
ATGTGAATACAAGTTTCGATCCCAGTGTTTATTCAAACCAGCAATGCATGAAGGCACTAA
CTTAAACCAACAACAGCCAACATGAACCCATTCAACTGGTGGCTATGAAGCCTTTTTTGT
TGGACATCAGCTGTCTATAAGTGACATGGCTTGAATGAGTGGTGGANATGCATTAATAAT
TTCACTATGTGTGTGTGTGTGTGTGTGTGTGTTTCTGTTGCCCCTCAAGTGTGTATCA
AAGTTACAATAATCAACTTCACAGTGACCAAGTCTGGCTTGGAGGACCAACT
>DLZ-415
ACAAAGCTGAATTTACAGTATATACCAGCATCTATACATAGAGATCAGTTTTAGATACGT
TAATAAAAAAAATCATCACATCTCTCCATCTCCCAACATCTGTTTCGTTTAATCAAAACCT
TTATTCAAATATAGGCATCAACTGAGACAAATGTCTTCACAAATACAAAAAAGTTAAATT
CTGAATGTTAAATCATTTGAAAATCCCTCTTGAGGTTTCACGTCTGTCGGGGAAATATTA
ACTTTCACCATCACTGCAGCTAANAACTTGAAATTTTGTAAATCCGCTACCTTATCAGAG
CATTCAACNTAAAAAAAGAATAATGGANAGAATGATTGCTACACACGCACACACACACAC
ACACACACACAGACNAGCACACACACGCACAGCCTTTATGAAAATGCCGCATAACAGCAG
T
>DLZ-453
CGAAGTTATAGGCTGAGACATACATGATCTTCTTGCCAACTTGAAGCCCAACTTGTCTTA
TACAAAGAGCTAGCTTAAATCTGTCTGTGTAGAAAATCAAAGAGACGATGTGCAGGANGC
TACTTGGAGAATCCTCCATCCTGCTANGTTGTTTGATTTCAAACAGCANGTGTGTATTAN
ATCCCCTACAGTTTAGGGGCTCTCTCTCATCCTCTATTCACACACACACACACACACACA
CCCACACACATAANATAATCAAAGGAAAAATCACCTGTCTCCTC

WP10: High resolution mapping of sea bream genome based on the BRIDGE-MAP map

Start date or starting event	1				
Activity type [1]	RTD/Innovation				
Participants	KULeuven (CR-12)	HCMR (CR-13)	UNIPD (CR-14)	BIOLUNIPD (CR-14.1)	CNRS (CR-16)
Total Person Months	1.3	26	6	15	16.11
Person Months allocated in 2nd year	0.6	10	2	5	-

Objectives

A) Further enrich with genes, including those involved in stress response and disease resistance/susceptibility, and microsatellites the medium to high resolution merged map of sea bream (BRIDGE-MAP), made up of a microsatellite-based linkage map and a radiation hybrid map.

B) Produce a comparative genomic analysis by further development of the BRIDGE-MAP database with maps and genomes of commercial and model fish and non-fish vertebrates, integrating on the map & the database co-expression information originating from the functional part of the project.

Progress towards objectives

D10.01: Extension of the BRIDGE-MAP linkage map to up 300 microsatellites (100 new)

We used all gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) ESTs which were unigenes downloaded from the MGE unigene site in February 2006. As of that date, there were a total of 18,196 sea bream unigene clusters containing 29,895 ESTs and a total of 17,716 sea bass unigene clusters containing 29,260 ESTs listed and annotated in the MGE database.

For each species separately, we used the ACE format of the Cap3 [Huang X & Madan A (1999). *Genome Res*. 9, 868-877] output file, the Annotation and FASTA-formatted text files and the file of all singletons to automatically generate, using a Perl script, three MySql tables: a "core" table with description of unigenes, an "Annotation" table containing the Annotation data and a and "seq" table with all unigenes sequences.

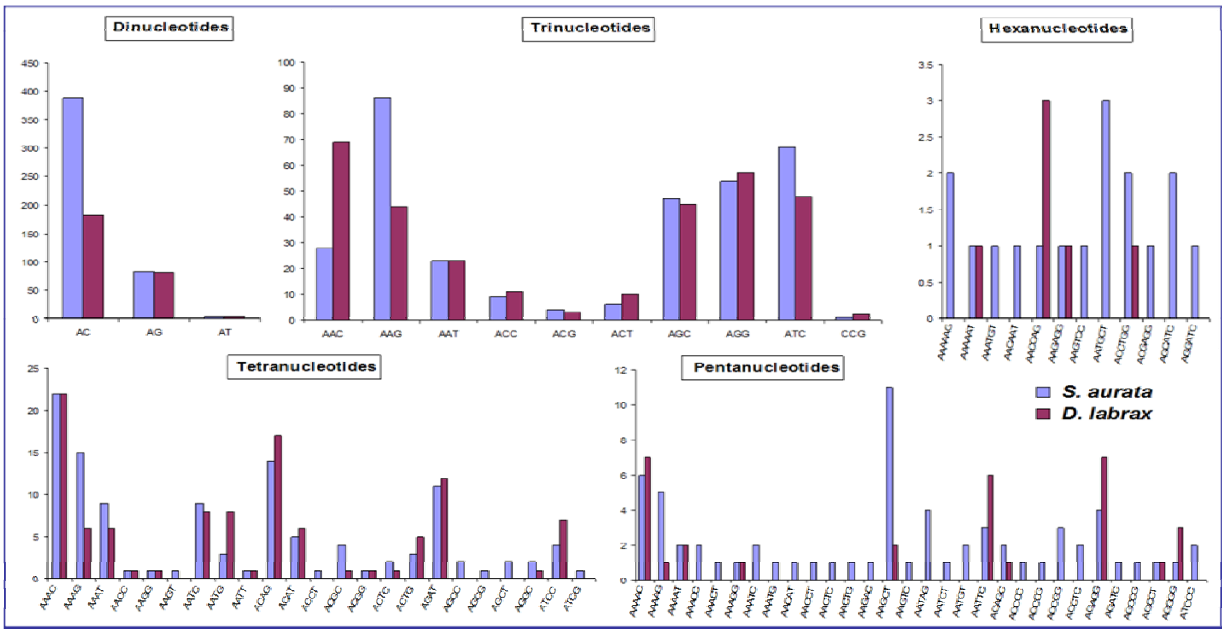
Both unigene databases were used to identify and characterize SSRs using a Perl script based on the algorithm of the MISA script (http://pgrc.ipk-gatersleben.de/misa). Perfect tandem repeats were defined in sequences with a minimum number of repeats of 9 for dinucleotide, 6 for trinucleotide, 5 for tetranucleotide, and 4 for penta- and hexanucleotide repeats. These results were added as a table in the data-set. SSR-ESTs analysis was performed with a set of SQL queries, and tables were automatically generated with Perl scripts; SSR-ESTs were analyzed for redundancy by Blast and/or Cap3 softwares.

Table 1: *Detection of SSRs found in sea bream and sea bass ESTs (SSR-ESTs). Number of repeats is considered 9, 6, 5, 4 and 4 for di-, tri, tetra-, penta- and hexanucleotides, respectively. Data for other fish are from Ju Z et al. (2005, In Silico Biology 5, 0041).*

Species	Unigenes	SSR-ESTs			Number of SSRs / motif					Total Nb SSRs
		Nb	%	Annotated	Di-	Tri-	Tetra-	Penta-	Hexa-	
Sea bream	18,196	899	4.94	284 (31.6%)	474 (47.6%)	325 (32.6%)	115 (11.5%)	66 (6.6%)	17 (1.7%)	997
Sea bass	17,716	705	3.97	321 (45.5%)	267 (37.6%)	312 (41.5%)	109 (14.5%)	47 (6.3%)	16 (2.1%)	751
Zebrafish	24,003	1,749	7.3	-	1,497 (64%)	579 (25%)	202 (8.6%)	55 (2.4%)	-	2,333
Medaka	8,158	209	2.6	-	105 (47%)	81 (36%)	27 (12%)	10 (4.5%)	-	223
Fundulus	16,726	369	2.2	-	237 (52%)	155 (34%)	43 (9.5%)	18 (4.0%)	-	453

Totally, we found 1,604 ESTs that harbor 1,748 SSRs in both species. The frequency of SSR-ESTs ranged from 3.98% for sea bass to 4.94% for sea bream and is in the range reported for model fish species which ranged from 1.5% in *Xiphophorus* to 7.3% in zebrafish [2.6% in medaka and 2.2% in *Fundulus*. In both species, the percentage of the annotated ESTs ranges from 31.6% in the sea bream to 45.5% in the sea bass.

Figure 1: *Distribution of SSR motifs in gilthead sea bream and European sea bass*



The dinucleotide repeat motifs are the most abundant SSRs in sea bream accounting for 47.5% of the SSRs [47%, 52%, and 64% for medaka, *Fundulus*, and zebrafish, respectively (Ju et al. 2005), followed by the tri-, tetra-, and penta- and hexanucleotide SSRs. Surprisingly, in the sea bass the most abundant repeats are the trinucleotides.

Among the dinucleotide motifs, AC/TG was the most abundant type in both species (Fig. 1) [also in all species in (Ju et al. 2005), except *Fundulus* in which AT/TA was the most abundant motif]. No CG/GC motif was found in sea bass and sea bream ESTs.

Ten only trinucleotide motifs were observed in sea bass and sea bream; the AAG and AAC motifs were the most abundant for sea bream and sea bass, respectively, whereas the other eight were approximately equal (Fig. 1).

We also evaluated SSR-ESTs distribution in the fourteen tissues used per species for cDNA library generation (Tab.2). Even though, in absolute numbers, SSR-ESTs are fewer in sea bass when compared to sea bream, in the former species 58% of the SSR-ESTs are tissue specific; this percentage is only 46% for sea bream.

Table 2: *Total number, type (di-, tri-, etc) and number of tissue specific SSR-ESTs (in light blue) per tissue for sea bream (SA) and sea bass (DL).*

Tissue	Total						Di-				Tri-				Tetra-				Penta-				Hexa-			
	SA		%	DL		%	SA		DL		SA		DL		SA		DL		SA		DL		SA		DL	
Liver	99	43	43.4	94	50	53.2	45	20	48	30	39	17	26	8	8	4	14	10	5	1	4	1	2	1	2	1
Ovary	81	37	45.7	89	54	67.5	33	16	29	13	34	13	46	29	8	3	9	9	4	4	2	1	2	1	3	2
Testis	92	48	52.2	92	55	59.8	36	18	28	18	37	19	40	24	9	5	12	6	6	5	8	6	4	1	4	1
Bone_Cartilage	122	58	47.5	84	48	57.1	64	29	26	15	40	22	43	25	9	5	12	7	6	1	2	0	3	1	1	0
Brain_Pit	123	68	55.3	64	39	60.9	58	38	29	15	45	20	21	16	10	6	10	6	6	3	2	2	4	1	2	0
Heart_Vessels	114	46	40.4	79	46	58.2	57	25	33	21	36	10	25	12	10	7	13	8	9	3	7	4	2	1	1	1
Adipose	101	44	43.6	86	48	55.8	39	17	27	15	39	12	41	26	15	11	12	5	6	3	4	2	2	1	2	0
Head_Kidney	124	54	43.5	62	33	53.2	64	34	19	8	40	13	30	17	13	5	9	6	5	1	4	2	2	1	0	0
Trunk_Kidney	96	35	36.5	81	40	49.4	40	15	26	12	35	8	32	17	10	5	13	8	9	7	6	3	2	0	4	0
Gill	106	45	42.5	81	47	58	46	23	33	20	41	12	26	14	8	4	10	6	9	5	7	3	2	1	5	4
Intestine	127	63	49.6	71	41	57.7	54	27	23	13	47	19	31	16	17	10	9	6	8	7	8	6	1	0	0	0
Spleen	123	66	53.7	80	45	56.3	51	33	26	14	45	19	37	20	14	8	11	7	9	5	5	4	4	1	1	0
Muscle	127	58	45.7	58	33	56.9	66	36	19	11	44	15	24	13	10	5	7	5	6	2	6	4	1	0	2	0
Skin	95	45	46.9	27	17	63	41	22	9	5	32	7	15	9	14	11	2	2	8	4	1	1	1	1	0	0

We used as template the non-redundant SSR-ESTs; for sea bream and sea bass, 91.32% (*i.e.* 821 out of 899) and 73.9% (*i.e.* 521 out of 705) of the SSR-ESTs, respectively, were non-redundant. As compound microsatellites were considered those present in the same EST and distant by a maximum of 25bp. SSR-ESTs were used as target for the Primer3 software (**3**) with a) primer length from 18 to 27 bases, b) primer's Tm ranging from 55 to 63°C, and c) product size in range of 100 to 250bp. The primers output of Primer3 was further analyzed in order to avoid or minimize the occurrence of tandem repeats in the primer sequence, the self and pair complementarity. This procedure was automated with a Perl script.

For the sea bream, there were finally 664 pairs of primers accepted (73.85% of the initial 899 SSR-ESTs). Primers were ordered for 260 sequences that approximately correspond to all those containing equal or greater than 15, 6, 6, 5 and 5 repeats for di-, tri, tetra-, penta- and hexanucleotides, respectively. When these primers were PCR checked on wild sea bream individuals, we saw that 78.1% of primer pairs gave PCR products of expected size in sea bream, 8.5% gave much bigger or non-unique products, and 13.4% did not amplify at all. Preliminary genotyping analysis with approximately 20 fluorescent primers, mainly for dinucleotides, indicated that more than half of them are polymorphic.

More specifically, data on 260 non-redundant EST-SSRs defined on sea bream's MGE database show that:

- 203 have a PCR band of expected size (78.08%),
- 22 gave a product of much different size (8.46%),
- 35 gave no product (13.46%)

Table 3: *Type (di-, tri-, etc) and number of EST-SSRs found in sea bream, for which there was primer design and positive PCR results.*

	Type of repeats in SSRs					
	Composite	**2-nucleotides**	**3-nucleotides**	**4-nucleotides**	**5-nucleotides**	**6-nucleotides**
Total	2	61	94	35	7	4
Annotated*	2	18	28	8	1	1

* In most cases annotation is like '*Tetraodon nigroviridis* (green puffer), chromosome undetermined scaffold XX, whole genome shotgun sequence...', i.e. those ESTs show significant Blast hit with sequences in other model fish but their function is yet unknown.

Furthermore, in HCMR there is an enriched DNA library for (AC)-type microsatellites from which some new sea bream SSRs were found and genotyped. There were also primers ordered from a recent publication on sea bream (Launey *et al*. 2003, Mol.Ecol.Notes, 3, 457-459). In total, during the second year (Annex 1):

- 13 EST-SSRs were found polymorphic on the linkage panel (out of 17 tested),
- 2 new markers from the HCMR's enriched library (out of 5 tested),
- 3 microsatellite markers characterized by Launey *et al*. (2003) were genotyped, and

- 2 markers mapped in sea bass were also genotyped on sea bream (out of 4 tested). Interestingly, the first one (DLA0064-cskiI) comes from a tilapia (*Oreochromis sp.*) annotated EST.

Currently, we are in the procedure of optimizing 9 new markers from the HCMR's enriched library, 7 microsatellite markers characterized by Launey *et al*. (2003) and 17 new EST-SSRs (Annex 2).

Additionally, in UNIPD (partner 14) seventy (70) new clones from a microsatellite-enriched genomic library were sequenced, and after sequence analysis 45 clone sequences contained a repeat greater than 18bp and were suitable for primer design. Those clones were used for "cold" primer design and 36 give good PCR products. When 36 fluorescent primers were ordered and synthesized for the above clones there were finally 22 informative loci that were informative and are going to be scored by January 2007.

D10.02: Extension of the BRIDGE-MAP RH up to 2000 markers (500 new) and a merged linkage & RH map reflecting recombination rates across the genome

In 2nd year, there was primer design that was performed batch wise, taking into account intron-exon boundaries for sea bream ESTs. The 1st Batch with 112 markers designed based on immune related genes downloaded from NCBI and generated in HCMR (Annex 3), and a 2nd Batch with 192 markers designed based on Medaka homologues (Naruse *et al.* 2006: A Medaka Gene Map: The Trace of Ancestral Vertebrate Proto-chromosomes Revealed by Comparative Gene Mapping) (Annex 4).

Table 4: *Markers genotyped on the RH map. Table source Senger et al. (2006) and Sarropoulou et al. (in preparation).*

Markers	Numbers of markers genotyped	Successfully PCR amplified	Number of markers on the actual map	Origin
TYPE I : Coding sequences	**840**	**694**	**497**	D.M.Power L.Bargelloni G.Kotoulas
Type II: Microsatellites	**560**	**463**	**448**	D.M.Power L.Bargelloni G.Kotoulas
Total	**1400**	**1157**	**945**	

Deviations from the project work-program

There is a major deviation from the work-program that has to do mainly with the generation and optimization of new microsatellite markers. Moreover, this delay seems to be under control since the use of EST-SSRs provides a considerably high number of polymorphic markers; in annex 1, we can easily perceive that EST-SSRs are more polymorphic and with higher number of alleles than the genomic SSRs. The same EST-SSRs could also be genotyped and enrich the RH map.

List of deliverables

Code	title	Due date (Month)	Actual/foreseen achievement date (Month)
D10.01	Extension of the BRIDGE-MAP linkage map to up 300 microsatellites (100 new)	12	30
D10.02	Extension of the BRIDGE-MAP RH up to 2000 markers (500 new) and a merged linkage & RH map reflecting recombination rates across the genome	24	30
D10.03	Comparative genome maps between of sea bream, sea bass, model fish species and human	32	38
D10.04	A final version of the BRIDGE-MAP database extended for comparative genome analysis	36	42

List of milestones

Code	title	Due date (Month)	Actual/foreseen achievement date
M09	sea bream RH map for identification of QTL genes	36	42

WP 10: ANNEX 1: New microsatellite (SSR) clones genotyped on the sea bream panel. Ms and E, in the first column indicate genomic SSRs and SSRs found in ESTs (EST-SSRs), respectively; shading shows annotated ESTs.

	ID_Clone Name	Repeat Motif	PCR_length	Nb alleles	Fluorescence	Polymorphism	Forward	Reverse
01	SauA25INRA	(TG)n*	152-170	7	FAM	pol	TACAAACAGCAGCATTAGAG	ACAAAGGATTATACAGAGCTACC
02	SauANINRA	(TG)18	153-163	5	TET	pol	TGTTGGAGCTTGTTGGTACAC	GAGCTGTAAACCGCTCAGG
03	SauD182INRA	(CA)9+(CA)8	234-252	7	HEX	pol	ATTCAAGACAGTATCCGACATG	TCTTCATCTCGTTTCACGTG
04	C1	(GT)13	215-219	3	HEX	pol	CGTGGCTTCTGTCTGTGTG	GACCACCCTCCCATAAGTTG
05	C12b	~(CA)14	222-236	4	TET	pol	AGGCTGGCTCTGCTAACC	GCCGAAACGAACAAGAAGTAG
06	DLA0064-cski-I	(AG)29	248-272	12	FAM	pol	Acc.Nb.AJ012011	Oreochromis, Dic.labrax
07	DLA0067-dlz070	(TG)32	162-180	6	TET	pol	Acc.Nb.DQ363888	Dic.labrax
01e	cDN09P0005I03.F.ab1	(ATAG)21	153-263	20	ROX	pol	TGGATCCGCTAATTCAGTTTC	AATTTCCCTGTCTGGGATCA
02e	CL219cng1	(GA)24	145-233	22	FAM	pol	TGACTTGTATAGGCAGCCAGAG	GATGCTCAGGAGCTACCGTC
03e	CL236cng2	(AC)24	135-183	17	HEX	pol	GTGCAGATGACCGAATGACT	GCCTTCAACCATGACAGATG
04e	cDN03P0001O12.F.ab1	(TTC)14	247-388	30	HEX	pol	ACATGACACTGCCTTCTGGA	AGGAGCAGGTCACGGAGA
05e	cDN08P0006E11.F.ab1	(TCTA)15	187-223	10	FAM	pol	GATCTGTCAGCTTACGTTCTT	CACCGTCTTGACAAGTGA
06e	cDN07P0006C15.F	(CA)25	175-227	21	FAM	pol	CCTTGAGCGATTGTTCCAC	CAGCCTTCAGCCATGACA
07e	CL2335cng1	(CA)30	177-223	18	TAMRA	pol	CACGGCAACAGACGAGAG	CCTCAGGCTAGAGAATGCATAA
08e	cDN06P0004J18.F.ab1	(AG)26	217-247	7	TAMRA	pol	GTGTGGCATACTTCCTCTGC	CCAGACGATTGGTACAGGTTC
09e	cDN11P0003K18.F.ab1	(CA)18cg(CA)10	180-220	18	HEX	pol	CCTTCTCCTTCCAGACATCC	GTCAGACACAGGCAGCCAT
10e	cDN08P0003D22.F.ab1	(AC)26	147-171	9	FAM	pol	CTGCAGACTGAACACACGC	TGTTAGATGGAGACTTCAGAGGTT
11e	CL3295cng1	(TC)12tttctgtctctcgctcag(AC)10	237-255	9	HEX	pol	GGTCTGATCTGTGGATAGGAA	CTCCGACAAGCTGATAACATA
12e	cDN07P0002P24.F.ab1	(CA)33	154-244	28	ROX	pol	TGTTCGCTGTGGCTATCG	CGGTGTGGATGTGACTCTGT
13e	cDN02P0001C17.F.ab1	(CA)36	272-338	24	FAM	pol	ATTGAGGTCAGCAACCGTG	TTCCAGAATCCAGCTCTGAAC
E	CL1639cng1	(GAG)6	127	1	FAM	mono	GACATCCAGGCATCTACGGT	CTGAGCCACGTTAACTGTTCTT
E	CL3910cng1	(ACA)8	123-204	8	HEX	pol/mono	CGAGAGCCTGGATGACAAG	GAAGATGTCGTTGTGGTTGG
E	CL2441cng1	(TCC)6	138-159	4	ROX	pol/mono	AGACTCAGAATGGTCGAGTGC	ACCTGTAGCTGGTGAAGCTG
E	cDN07P0003D01.F.ab1	(AGC)8accggtggaactagtcttca(CAG)7	167-183	4	HEX	pol/mono	GTCTCAACGTGATGTCCTGC	GCTGTGAAGACTAGTGCCACC
Ms	DLA0070-dlz207	(AC)30	105-131	6	HEX	pol/mono	Acc.Nb.DQ363891	Dic.labrax
Ms	DLA0059-dlz047	~(AC)23	238-328	3	HEX	pol/mono	Acc.Nb.DQ363882	Dic.labrax
Ms	C7	~(AC)17	205	1	FAM	mono	CCTCCTCTCCAAAGGTCTCC	GATCGCACAGTCACACATCC
Ms	D12b	(CT)13	192-202	5	FAM	pol/momo	CTCGCCGTCTCTGTCTATCC	TGGACTGTACTGGGCTGAAC
Ms	G4	(GT)13	206-222	5	TET	pol/momo	AGCCTGTCTGCTTATCAACG	AATCGCTCACTTGCTCACTC

WP 10 ANNEX 2: New SSR clones currently optimized for genotyping on the sea bream panel. Ms and E, in the first column indicate genomic SSRs and SSRs found in ESTs, respectively; shading shows annotated ESTs.

	ID_Clone Name	Repeat Motif	PCR_length	Fluorescence	Forward	Reverse
Ms	C11	(AC)15	123	FAM	TACCACTCCTCTCTGTCC	TGCAGGGTCTGTTATTAA
Ms	D12a	~(TC)16	113	FAM	TCGTTCTCCACCTGAGTTCC	AGAGGAAGAGGGAGGGACTG
Ms	E12	(CT)19	223	TET	AAAGAGAGAGCGCGTGAGG	GAGAGGCAAGGTCATTGTGG
Ms	E6	(GT)22	166	TET	GGCCTGGTTCTCTTACTTTGC	CAGCTCATTTGGCCTTTG
Ms	F7a	(CA)18	175	FAM	AGCATGTGTGCAGGTAATGG	TTTGGTGGTGTCTCTCAAACC
Ms	G2	~(GT)21	230	HEX	ACCACAGAAAGTCATAAACA	ATGGAAACCACACTAACG
Ms	G6	(AC)23	180	HEX	TGGGTCAGGATCTCATGTTTC	TTGTGTGTGTGTTTGCAGTTT
Ms	G6tetra	(TTTC)15	148	TET	GCCGCGAAGATAAGGAAATA	AGGGAGACAGAGGGAAGAGG
Ms	G9	(GT)19	220	HEX	CCCATCCATCTTTCCCTCTC	GCCGATAAAGGAGGTCACAG
Ms	SauD69INRA	(TG)19T(TG)7	144–187	FAM	CGTTGATCCCTGAGAAGC	AATACACGGAGAGCCACTG
Ms	SauE82INRA	(CA)12AA(CA)7	159–183	FAM	ATTGGGTGGCAGTTTAGTAGG	CACTGCGATGAGTGACCC
Ms	SauE97INRA	(CA)30	168–196	HEX	ACATTCATGTGTAAAATCGG	TTGGAAGAACAGAAATCTAATG
Ms	SauG46INRA	(GT)6(GA)6GGAA(GA)8(GT)19	152–202	HEX	GTGAACACCTGCCAGACG	GCATCGAGGTCAAGTACCTG
Ms	SauH94INRA	(CA)12N19(TG)7	152–212	TET	GTCTGAATGTTCCCATAGCTC	GCCACAGCTGTAACTCACTC
Ms	SauI41INRA	(CA)9CG(CA)4CG(CA)13	182–233	FAM	AACAGTTTGTGATTATTCATCG	CACGTCTAACCTGTGATTAGC
Ms	SauK140INRA	(CA)23	145–165	HEX	TTTCACTGAGCTGGAGACTTG	AGAGTTGAGTCTGTTGCATGC
E	cDN12P0001M10.F.ab1	(TTTTC)11	137	FAM	GCCAACCTAATATGCCACC	AATGGCACGGAGTACAGC
E	CL3420cng1	(GT)9gc(GT)15	197	FAM	TTCTGTCGTGGAGTTGCTTC	TTCACACTGGCCTTAATCCTC
E	CL3102cng1	(CA)20	228	FAM	TGATCTGTTCGCTCATCCAC	AGCTGAAGCCTACCTGAACC
E	cDN11P0004B17.F.ab1	(GCTAA)6	248	FAM	CCATCAACATGTGAGTTAAGTAGA	AGCCTGGAGACAAGAGACAG
E	CL2416Contig1	(ATCT)9	175	FAM	TTCTGTCGCTTGACTCATT	GAAGTGAACCTGTCAGTGGTA
E	cDN08P0004J06.F.ab1	(TGGACC)5	232	HEX	TCCTGAAGTTCCTGAAGCG	GCAGCCTGGTGATGTCATAG
E	cDN05P0003C12.F.ab1	(CTT)11	162	HEX	GCATGGCAGCTGAACTGT	CTCGAGTGGATACAAGCCG
E	cDN13P0005H11.F.ab1	(GCA)10	178	HEX	AGACTGGAGTCTGTCCTGGAA	GGCCTCACAGGTCACAGAG
E	CL1330Contig1	(GGA)10	198	HEX	GATGAACTGACCTGGCTGC	TGGCATGCTCTTGATGTGA
E	CL2163cng1	(CA)42	213	ROX	TCACTGACATCGCCACCT	CTGCTCACTTCCTGTGTCAA
E	cDN09P0005N05.F.ab1	(ATAG)12	165	ROX	CATCAAGTTCAGCATCAGC	AAGTGAACCATGACTGTCAGA
E	cDN06P0001M14.F.ab1	(CGAGGA)5	236	ROX	GTCGGAGGCAGACTCCAT	CAGAGCTTCAGAATCAGCGTT
E	CL4030Contig1	(GGGTC)6	190	ROX	GAGCTCACCTGTGTCACTGC	GAGACGTCACCTGTCCACC
E	CL1275cng1	(TG)9(AG)18	197	TAMRA	GGTGGAGCACAGGTTACAAG	GTCACGTCTCATCTACGTGC
E	cDN14P0001H06.F.ab1	(TTTC)13	224	TAMRA	TTGGACTGCCTCCACACTC	CAGCTGCTCTCTAACTCAGCA
E	cDN04P0002A13.F.ab1	(GA)24	167	TAMRA	TGACTTGTATAGGCAGCCAGAG	GATGCTCAGGAGCTACCGTC
E	CL324cng1	(AC)22	244	TAMRA	TCTCCATAACATCCATGCCA	ACACAATTGTCGAACAAGCAG

WP 10 ANNEX 3: Genetic markers genotyped on the RH map and designed based on immune related gene sequences either downloaded from NCBI or generated in HCMR.

Serial #	Gene names	Species	Source
1	aryl hydrocarbon receptor 2 (AHR2) (partial)[SA] /partial cds/	Sparus aurata	NCBI
2	activated protein kinase C receptor[SA] /partial cds/	Sparus aurata	NCBI
3	arylalkylamine N-acetyltransferase (AANAT1)[SA] /complete cds/	Sparus aurata	NCBI
4	calmodulin mRNA (partial)[SA] /partial cds/	Sparus aurata	NCBI
5	cathepsin D[SA] /complete cds/	Sparus aurata	NCBI
6	cyclo-oxygenase-2 (cox-2 gene)[DL] //	Dicentrarchus labrax	NCBI
7	cytochrome P4501A1[SA] /complete cds/	Sparus aurata	NCBI
8	Endogenous antimicrobial peptides (AMP) dicentracine mRNA[DL] /complete cds/	Dicentrarchus labrax	NCBI
9	estrogen receptor[SA] //	Sparus aurata	NCBI
10	glucocorticoid receptor (1)[DL] /complete cds/	Dicentrarchus labrax	NCBI
11	glutathione S-transferase (partial)[SA] //	Sparus aurata	NCBI
12	GnRH receptor (partial)[SA] //	Sparus aurata	NCBI
13	gonadal P450 aromatase[SA] /complete cds/	Sparus aurata	NCBI
14	growth hormone[SA] /complete cds/	Sparus aurata	NCBI
15	heat-shock protein 90 beta mRNA, partial[SA] //	Sparus aurata	NCBI
16	hormone receptor type II + GENE[SA] /complete cds/	Sparus aurata	NCBI
17	IL-1b gene for interleukin-1 beta, exons 1-5[SA] //	Sparus aurata	NCBI
18	immunoglobulin light chain[SA] /partial cds/	Sparus aurata	NCBI
19	insulin-like growth factor 1[SA] /complete cds/	Sparus aurata	NCBI
20	insulin-like growth factor 2[SA] /complete cds/	Sparus aurata	NCBI
21	interferon regulatory factor 1 (IRF-1)[SA] /complete cds/	Sparus aurata	NCBI
22	interleukin-1-beta (il-1b gene)[SA] //	Sparus aurata	NCBI
23	larval cDNA similar to receptor for activated protein kinase C[DL] //	Dicentrarchus labrax	NCBI
24	lectines (FBP32)[Morone chrysops] /partial cds/	Morone chrysops	NCBI
25	macrophage colony stimulating factor receptor (csf1r gene)[SA] //	Sparus aurata	NCBI
26	metallothionein[SA] /complete cds/	Sparus aurata	NCBI
27	MHC class I alpha antigen (Spau-UA) mRNA[SA] /Spau-UA*02 allele, complete cds/	Sparus aurata	NCBI
28	MHC class II alpha antigen (Spau-DAA) mRNA[SA] /Spau-DAA-101 allele, complete cds/	Sparus aurata	NCBI
29	mRNA for CD8 alpha chain [DL] //	Dicentrarchus labrax	NCBI
30	mRNA for estrogen receptor beta 2, clone 21[SA] //	Sparus aurata	NCBI
31	partial mRNA for estrogen receptor beta 2 (clone 54)[SA] /partial /	Sparus aurata	NCBI
32	Mx protein[SA] /complete cds/	Sparus aurata	NCBI
33	natural resistance-associated macrophage protein (nramp gene) (partial)[SA] //	Sparus aurata	NCBI
34	nonspecific cytotoxic cell receptor protein-1(NCCRP-1)[SA] /complete cds/	Sparus aurata	NCBI
35	parathyroid hormone-related protein precursor (PTHrP)[SA] /complete cds/	Sparus aurata	NCBI
36	putative iodothyronine deiodinase type 1 (dio1 gene)[SA] //	Sparus aurata	NCBI
37	T cell receptor alpha (TCRalpha) mRNA, partial[SA] //	Sparus aurata	NCBI
38	TLR9 isoform B[SA] /complete cds, alternatively spliced/	Sparus aurata	NCBI
39	TNF-alpha gene for tumor necrosis factor alpha, exons 1-4[SA] //	Sparus aurata	NCBI
40	transforming growth factor beta 1[SA] /complete cds/	Sparus aurata	NCBI
41	tyrosine hydroxylase mRNA, partial[SA] //	Sparus aurata	NCBI
42	vitellogenin (partial)[SA] //	Sparus aurata	NCBI
43	thyroid hormone receptor-beta (THRb)[SA] /complete cds/	Sparus aurata	NCBI
44	similar to CC chemokine-1	Ictalurus punctatus	NCBI
45	similar to Complement C1q A chain precursor	Ictalurus punctatus	NCBI
46	similar to complement C4B	Ictalurus punctatus	NCBI
47	similar to Complement subcomponent C1q chain B precursor	Ictalurus punctatus	NCBI
48	similar to Cyclophilin D	Ictalurus punctatus	NCBI
49	similar to Embigin protein	Ictalurus punctatus	NCBI
50	similar to Galectin like protein	Ictalurus punctatus	NCBI
51	similar to I kappa B (IkBL) protein	Ictalurus punctatus	NCBI
52	Icpu-A-2 MHC class II antigen	Ictalurus punctatus	NCBI
53	similar to clone PG7 sterile class G IgL chain	Ictalurus punctatus	NCBI
54	similar to MHC class I alpha chain	Ictalurus punctatus	NCBI
55	similar to MHC class II beta chain	Ictalurus punctatus	NCBI
56	similar to Ig rearranged H-chain mRNA V-C-region	Ictalurus punctatus	NCBI
57	similar to immunoglobulin heavy chain	Ictalurus punctatus	NCBI
58	similar to immunoglobulin light chain F class	Ictalurus punctatus	NCBI
59	similar to NADH dehydrogenase (ubiquinone) 1 beta subcomplex	Ictalurus punctatus	NCBI
60	similar to Interferon consensus sequence binding protein	Ictalurus punctatus	NCBI
61	similar to Interferon-induced protein 1-8D	Ictalurus punctatus	NCBI
62	similar to Homology to murine Mx genes	Ictalurus punctatus	NCBI
63	similar to LUCT/interleukin-8	Ictalurus punctatus	NCBI
64	similar to Danio rerio invariant chain-like protein 1 (Iclp-1)	Ictalurus punctatus	NCBI
65	similar to Monoclonal non-specific suppressor factor beta	Ictalurus punctatus	NCBI
66	similar to Fc-epsilon-receptor gamma-chain protein precursor	Ictalurus punctatus	NCBI
67	similar to B-cell receptor-associated protein 31	Ictalurus punctatus	NCBI

ANNEX 3 (cont.):

Serial #	Gene names	Species	Source
68	beta-2 microglobulin [Paralichthys olivaceus]	Paralichthys olivaceus	S.aurata HCMR
69	CCL4 [Oncorhynchus mykiss]	Oncorhynchus mykiss	S.aurata HCMR
70	complement component C3 [Paralichthys olivaceus]	Paralichthys olivaceus	S.aurata HCMR
71	complement component C7 [Paralichthys olivaceus]	Paralichthys olivaceus	S.aurata HCMR
72	granulocyte colony stimulating factor receptor [Oncorhynchus mykiss]	Oncorhynchus mykiss	S.aurata HCMR
73	pentraxin [Oncorhynchus mykiss]	Oncorhynchus mykiss	S.aurata HCMR
74	perforin [Fundulus heteroclitus]	Fundulus heteroclitus	S.aurata HCMR
75	myeloid differentiation factor 88 [Oncorhynchus mykiss]	Oncorhynchus mykiss	S.aurata HCMR
76	aquaporin 1[SA] /complete cds/	Sparus aurata	NCBI
77	aquaporin 1-like[SA] /complete cds/	Sparus aurata	NCBI
78	aquaporin mRNA, complete[SA] //	Sparus aurata	NCBI
79	Prolactin receptor mRNA, complete cds	Sparus aurata	NCBI
80	prolactin (PRL) mRNA, complete cds	Sparus aurata	NCBI
81	Histone H2A and H3 genes	Rainbow trout	NCBI
82	histone H4 and H2B genes	Rainbow trout	NCBI
83	histone H1-0 (H1f0) mRNA, partial cds	Oncorhynchus mykiss	NCBI
84	cytochrome b (cytb) gene, partial cds; mitochondrial gene	Sparus aurata	NCBI
85	Cytochrome c oxidase subunit I mRNA, partial cds; mitochondrial	Sparus aurata	NCBI
86	Fructose-1,6-bisphosphate aldolase mRNA	Sparus aurata	NCBI
87	Similar to High affinity immunoglobulin epsilon receptor, mRNA sequence	Atlantic Salmon	NCBI
88	Thymosin beta mRNA, complete cds	Oncorhynchus mykiss	NCBI
89	Lysozyme type II gene, complete cds	Oncorhynchus mykiss	NCBI
90	Myosin light chain 2 mRNA, complete cds	Sparus aurata	NCBI
91	Fast skeletal myosin light chain 3 mRNA, complete cds	Sparus aurata	NCBI
92	Myosin heavy chain-like mRNA sequence	Sparus aurata	NCBI
93	Skeletal troponin T mRNA, complete cds	Sparus aurata	NCBI
94	Slow troponin T 1 (sTnT1) mRNA, complete cds	Sparus aurata	NCBI
95	slow troponin T 2 (sTnT2) mRNA, complete cds	Sparus aurata	NCBI
96	Heat-shock protein 90 beta mRNA, partial cds	Sparus aurata	NCBI
97	similar to Heat shock protein HSP 90-alpha CDNA, mRNA sequence	Dicentrarchus labrax	NCBI
98	similar to Heat shock protein 8 cDNA, mRNA sequence	Dicentrarchus labrax	NCBI
99	similar to Heat shock protein 70 cDNA, mRNA sequence	Dicentrarchus labrax	NCBI
100	Glyceraldehyde 3-phosphate dehydrogenase, complete cds	Oncorhynchus mykiss	NCBI
101	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, complete cds	Oncorhynchus mykiss	NCBI
102	Similar to olfactomedin 4 precursor [Danio rerio]	Danio rerio	D.labrax HCMR
103	alpha-2-HS-glycoprotein [Platichthys flesus]	Platichthys flesus	D.labrax HCMR
104	alpha-2-macroglobulin [Sparus aurata]	Sparus aurata	D.labrax HCMR
105	antifreeze protein precursor [Myoxocephalus octodecemspinosus]	Myoxocephalus octodece	D.labrax HCMR
106	CC chemokine CK-2.1 [Oncorhynchus mykiss]	Oncorhynchus mykiss	D.labrax HCMR
107	complement component C9 [Takifugu rubripes]	Takifugu rubripes	D.labrax HCMR
108	C-type lectin [Fundulus heteroclitus]	Fundulus heteroclitus	D.labrax HCMR
109	galectin [Paralichthys olivaceus]	Paralichthys olivaceus	D.labrax HCMR
110	heat shock protein 60 [Pseudosciaena crocea]	Pseudosciaena crocea	D.labrax HCMR
111	MHC class II protein [Morone saxatilis]	Morone saxatilis	D.labrax HCMR
112	growth hormone receptor[SA] /complete cds/	Sparus aurata	NCBI

WP 10 ANNEX 4: Genetic markers genotyped on the RH map and designed based on Medaka homologues (Naruse *et al.* 2006).

	Marker name	Forward	Reverse
1	cDN01P0001E03	TGTGGATGAGAGTCGCTACAA	TGAGCACGTTACCTGGAGAA
2	cDN01P0001I11	ACTGGAGCGACTTATCAAGACA	TTCTCAGTCATTGTCCACGC
3	cDN01P0001L10	CCGAGTATCTGAAGGACCG	CAGGTCAGCGTGACGAAG
4	cDN01P0002K06	TCAAGATGAGGAACGACCG	GGATCATGTTGAGGTGCTGA
5	cDN01P0002L12	CTGCAACACCTCCATGGTT	TGGTGATCAAGATCTCGCC
6	cDN01P0003A21	AGCTGTGCGAGACCTGAAG	CTCTGAAGACCACGTGTTGG
7	cDN01P0003C14	CCTGTGCTATGTTGGAGCC	CACAGCATCCAATGAGCC
8	cDN01P0003I15	AACATCCACAAGCGCATC	ACTTGCGGATCTCCTTGAT
9	cDN01P0003J20	GCCAAGGAAGCAGGTGAA	TGTCCATATACTTGGCGCTC
10	cDN01P0003L08	GGAATAACGGTCGTGCTAAGA	GACGCTGGCCTCTGTGAT
11	cDN01P0003O16	GGTCCAAGACCTCACTCCTG	ACAGACGCCTCCACGTCTA
12	cDN01P0004B02	GGCAGACGGTGATTGGTACT	TACTCGGTCACGTACTTCCG
13	cDN01P0004J02	TCTTCCTCTGCAGACACCAA	AAGACCAAGTCCTGAAGCCA
14	cDN01P0004L04	GATTCAGATGCATCCACGTT	CTCCTTATCCAGATCGAGTCC
15	cDN01P0004P04	CCTGACGTCTACGAAGCCT	CTCAATCCAGTAACCATCGG
16	cDN01P0005F03	CGACATCGACCTCAACAAGA	CTGGCGAGGATTCTGCAT
17	cDN01P0005H13	ACGCCGACTACCAGTACCTC	TAGAAGCCTCCGATCACCA
18	cDN01P0005J23	AGTGTCGTTTGCACAACCC	CTTTCCAGAGACCACGCAT
19	cDN01P0006B15	GCTCCAGACAGTAACCGCA	TGCTCGAAGTAGCAGATGGA
20	cDN01P0006D03	GGCAAGGTAATTGAGCAGATG	TAACGATCTGTTCCTGCGG
21	cDN01P0006I17	AGCTGAACCTCTGTGCATGA	TGCTGAGAGTGATCTGAACCTT
22	cDN01P0006O02	CCTGTGGTGAAGGATCCAA	TATGCGTCAGCGATGGTAA
23	cDN01P0006O20	CAGATTGTGCAGGTTCTTGG	CGTCAGGCTCCACATTGAT
24	cDN02P0001L05	ATTCCGTCATCCACAGAGGT	TGCAGGTATCCATAGATGAGGT
25	cDN02P0003C11	GAAGGATAATGACCTTGCTGC	AGCCAACTGGCTCTTCACAT
26	cDN02P0003M14	AACTACTGGATGTGGAGGAGG	GTACACCGCTTGCCTGAA
27	cDN02P0004A14	TTCTGCTGGAGCCATACAAG	AGCGTGCTAATGATGACGAG
28	cDN02P0004L17	CCATAACTCGTAACGCAGG	CATACTGATCCATCTGGAAGTC
29	cDN02P0004N04	TGGCACAGTATCCATTCGAG	TCTCCGTAGAAGTCCAGCG
30	cDN02P0005N02	TTGACGTTGTCCAGTTCGAG	CCACCGATGCAATGATACA
31	cDN02P0006C02	CTGCACACGGAAGACCAG	CGTAGCGAGCCTCATACCA
32	cDN02P0006C04	AAGGACCATGAAGCTGAGGT	ATGAGTGCTGTGCGGTTCT
33	cDN02P0006G21	TTGCTACACCTCGGTCCTTA	TGATGGTCTGATAGAAGTCATGC
34	cDN02P0006N14	GCAGCGTGGGTGTAATGG	TCTCCCTCTTTGGTGTCGTG
35	cDN02P0006N14	GCTGCGACGTAACTTCAATG	CTTCAGGCTCAGCAACTCCT
36	cDN03P0002D19	GAGCATCAGCTGGACGTTAC	AGTACTTGCAGTCCGCAGC
37	cDN03P0002G13	ACAACCAATACGGCGACA	CCGAAGGACGAAGCGTAG
38	cDN03P0002I24	AAGCAGCAAGCCATCGTA	GCGTTGAAGAAGTGCAACA
39	cDN03P0002L09	TCAGGCTACAGAACGACCG	GTGCAGGCGGAACTTAGTG
40	cDN03P0003D02	GGAGGTCATTGAATTGGAGG	GGCCTTGAGGTGAACTCTG
41	cDN03P0003H21	TAGGAGCGAGGTGGACCTTA	GGCAGCGTTGCAGACTTAT
42	cDN03P0003L22	ACATCTCAGCCACACCGAC	GGAATGTCAGAGTCCTCGC
43	cDN03P0003P17	GCCAACTCTGATGGCACA	ACACTCTCCCTTCACCCAGA
44	cDN03P0004H20	GCTGTATGAGAGGAAGCTCCA	GACCAGCCATGAGGATCAA
45	cDN03P0004L05	ACGGATCCTGTACGAGTTCAT	GATCCATCTGCCAGGATGTT
46	cDN03P0004L08	AGTCAACACGCAGGAAGATG	GGCAGGTCTGAACTGGTGA
47	cDN03P0004P12	GAGGCCTGACTGCATCTTATG	AGACTCAGGAAGTCCGAAGG

48	cDN03P0005E06	CAACGTCAAGACCATCCTCA	CCAGCTTCGGTAACTATCGC
49	cDN03P0005L10	TGCTTGTGTTGGACATGTTG	CCAGAGTGTGGACAGAGTGC
50	cDN03P0006E09	TGTGTATTCTCTTCACGGCG	TGTGCTCACGAGACTCTGCT
51	cDN03P0006F11	ACTCTGCTGGCAATCACAA	ATGGACACATACTCCATCGG
52	cDN04P0001J09	AATGACGACAAGCACCTCCT	AGCAACTCGCTGGATTCTG
53	cDN04P0001N21	TGCCAGTGAAGTGGATGG	TCATGTGTGCAGTTGGAGG
54	cDN04P0002B10	CTCCATGACAAGATCTACTGCG	CACGATGAGATGTGCTGACA
55	cDN04P0003N22	CATTGGAGCAGAGTCTAGACAAG	GAAGGTTGCAGTCAGCCAG
56	cDN04P0004D22	CGTTCACCAGTGGACTATGG	GCAATGATCTTGAGCACCTG
57	cDN04P0005B05	GCAGACCTTCGTTCCAACA	CTGTTCTGTACAGTCCGGTCC
58	cDN04P0006O14	GAAGGCATTCGTCCAGAAGT	AGGTCAGACCGCTCATTCTT
59	cDN05P0001B22	CTCAGCTGAACATTGGCA	TTGGCAGAAGCGATGACT
60	cDN05P0001I12	AACCTGACTGGTTCCTTGAGA	CCAACATCATCTTCTGTTGAGC
61	cDN05P0002F02	GTGGACAATCCGTCCTGTG	CCTGGACTCTGACTCGACCT
62	cDN05P0002F16	TCATGGAGTCTTACGAAGTGGA	TGTACTGTCCGATGGAGTGG
63	cDN05P0003H01	TCGTAAGGACGAAGGCTCTC	AACGGATGGCACAGAATGT
64	cDN05P0003M17	AACTGCCAGAAGCTGCCTA	TCTGATGTAGACGTTGGTCTGAA
65	cDN05P0004O04	CGTGAACATGTGTTGCCATA	CAGAGCTGAACGATAGATTGGA
66	cDN05P0005E15	CCAGATCACTCCAGAGCAG	GAGGCTCTCCTTCAACCAT
67	cDN05P0005J23	GGTGAATACTCTGAAGCCGC	TGCACCAGTAGAGATGACAGG
68	cDN06P0001H02	AGATCCCGATCTTGATTTCAC	CCGGTCTTGGAAGTGGAC
69	cDN06P0002F24	GACCGGAGAGCAGTACATCC	CATGAAGCCGCTCAGACA
70	cDN06P0002N05	CAACTAACGCCAACACCTCA	TATCAGCCACAGTCGTGGAC
71	cDN06P0004B14	GCTTGAGGATGAGAAGTCCAG	GGAGACATTGAGATGACAGAGAAG
72	cDN06P0004B21	TTGAAGGCTATGAGATGGCA	GATGAGGTTGGCGAGGAA
73	cDN06P0006C24	GTCCGTCTACGAGCTGGTTC	GGACTCGGTCCAGGTTGA
74	cDN06P0006I09	AGTGATGTAGCCAGAGGCAA	ACTCAGCCAGAGGAATACGC
75	cDN07P0002B03	CTCATCGTGGCCTACTGCTA	CCATTGTCCAAGGTCTCTAGTTC
76	cDN07P0002B22	TGATTCAGACTTCTTCGCCA	TGTGCAGTTCAGCGCATAC
77	cDN07P0002E24	GGTCATCTTGGTGAAGTGCT	CTCATCTCGCCATCTTCTTC
78	cDN07P0002I07	TGGACCTTGTCCAGTCTGC	CCTCAGACAGCTTCCTGGTT
79	cDN07P0002L06	GGAGCTCTTCGAACGCAT	TGATCCGTGTACCGGTTGT
80	cDN07P0002O22	GACGTGTGCATACCTGTCC	CTTGAGGTCTATGAGCAGGC
81	cDN07P0003A04	GCAATCCTGCCTCAGCTC	AGGACAGTCTGGACCACTCC
82	cDN07P0003P19	GTCCAAGGTGCACGACTT	AGTCATCTACAGCCTGTCCG
83	cDN07P0004A03	CCGATGGCGTACTCTTCC	CTAGCAGTGGTGGCTCGTC
84	cDN07P0004G13	CTCTCCGGTGACCAAGACTC	CAGGACTGCCTAGATGTCCA
85	cDN07P0004N19	TTGGCTCGTAATGACATTGG	GCAGGATGGAGTCCTTGG
86	cDN07P0005E09	ACAAGTTCACCAACGGAGG	ATGTACCACTGCCACGGAA
87	cDN07P0005M03	CCAGACTTTGTCCTGATTCG	CATGGCTTGTCACACAGGTT
88	cDN07P0006E09	AGGATCCGCAGGTTCTGTT	GAGGTCAGTGATGGCGTTC
89	cDN07P0006E23	TCACCAAGGATGAAGGCAC	TCTGTAGGCCAATCACGTTCT
90	cDN07P0006F01	GCCTCGCAAGATTGAAGAA	TCTCCTTGTCTGTGATGACCA
91	cDN07P0006L06	CATGGACCGCAACTCATACT	ATTCCAGGTAGCGAGTCACC
92	cDN07P0006M12	ATGTCAGTGGAACCAATCCA	CTGCAGCTCACTGAAGGAAC
93	cDN07P0006O14	GTCCGCCAAGACATTGAAG	TCCACTGGATTGTCAGCAATA
94	cDN08P0001D06	GGCATACCTGATGCTCAGAAG	GGAATTGAAGCCGTAATGGA
95	cDN08P0005J15	AGACATGTTCACTGCAACAGC	AGACAGGAACTTCTGCCAGG
96	cDN08P0005J24	TGGCAAGAATTCTGCATGAC	AGGCATCAAGGAAGTTCCAA
97	cDN08P0006B18	TACATCACTCTGGTCCGGTG	TTCAGTGCAGGATCTCGATG
98	cDN08P0006E16	AAGAGACAAGGACGCTCAGG	GTTGATGCCGCGGTAGAT

99	cDN09P0001I01	ACCATGGCGGAGCTAAGTT	GCCAGAATTGCTATCAACCA
100	cDN09P0001J16	ATGCCTTCGAGCACATCA	CGAGTCTGGTCGGACAAC
101	cDN09P0002E08	GAGGTGCTCATGGTATCATTGT	TCACTGGCATAGCGATCAAT
102	cDN09P0002O13	AGAGACCAGCACGACACCTT	CTGTGAACCGAAGCCTCC
103	cDN09P0003C05	CCGCCACTCTGTCATCTTC	ACTGCCATCTCGATCTGCTT
104	cDN09P0003F20	CAACGAGACCGTTGAAGACA	GAAGTTCTGGCGAACACGA
105	cDN09P0004K23	CATGCTACAACCTCGGAGTG	TCATGTAGGAGCCGTCTGG
106	cDN09P0004O22	ACATAAGTGCGGTGCCAA	TCATGAGCTTCTGGTCATCTG
107	cDN09P0005C15	TAATTGACGCTCCTGGTCAC	CGTCAGGTCCATCTTGTTCA
108	cDN09P0005N14	CGATGTTCGGCAACATTCT	ATAGCTCCATCTGAGCTGCC
109	cDN09P0006A02	TCAGGATATGGTTGTGGTTCTG	AGTTGTGAATGTGCTCCTGG
110	cDN09P0006M11	TTCTTGTCCTTCAACTCGTCC	CATGGCTTAAGTCCAGACTGC
111	cDN09P0007G17	CCAAGGCCTGTAATCTGG	TTTGATCATGGTCTCCAGC
112	cDN10P0001F24	CTTCAGCTTACAGACGCCAA	TGAACTTCTGCAGCTCGGT
113	cDN10P0001M15	CCTGTACATAGAAGCGGACG	TCCGAGTCAAGAGGCTGTAAG
114	cDN10P0002L06	CCGTGTTGCCTACGTCTCT	GCATGGATTGCCTCTTCAC
115	cDN10P0003C07	ACAAGCTGGACGAGGCAC	CCGTCATACAAGTGGCAGAA
116	cDN10P0003H19	TCTGGTGTAGCGGAGTCTTG	CGTCCTCGCAGTCACAGTAG
117	cDN10P0004B08	GCTGGCCAAGACTGGTGT	AGCTCTACTGAGGTGGCTGC
118	cDN10P0004E16	GCTGTCAGTCCAGACTGTGATA	TTGTCCTTGCCAGGATACAT
119	cDN10P0005C21	TTGGCCAAGGTCAGACATATT	TCGTCATTCTCGAAGTCAGC
120	cDN10P0006K17	GGTCGAAGACGTCAAGTTCC	CCGAGGATCTGTCCTGATG
121	cDN11P0001A02	CCGCATCATCTATGACAGG	TCTTCCTCCTCTGCCTCTTC
122	cDN11P0001P21	CTTCCGAATGCCATGAAT	GCTGATTAGACAACCAGTCCA
123	cDN11P0003O20	CTTCTGGCTTACTCTGGTTACG	CATGCAGTCCATGAGTATGAA
124	cDN11P0004K10	AGAGTGGATTGACAGGCG	AAGATGAAGGTATAGTTGCTGTTG
125	cDN11P0004L03	CCTGTTGAAGATGGTGATGC	CAGCCTGAGCGTTGACTC
126	cDN11P0004O13	AAAGTGAAGCAGGAGGGTTT	TCTTCAGGGTCGATGAGTTT
127	cDN11P0004P15	TCTTCATCCGAAGCTAAGGC	ATCGTCGTGGAGGATCAGA
128	cDN11P0005K16	AAGGACGGTCACCTGGACT	TCTGGATACCGAGCCTTCA
129	cDN11P0006I01	TCCGCCTTCAATACTCAAGAA	CAGCTCCAGCTGGTATCGTA
130	cDN11P0006P11	AGCAAGCCCAAAAAGAAGAA	CATGAGTCAGTTGCTCCAATG
131	cDN12P0001D04	GCTCCAATCCTTATCGCTACA	GCCAGTTGAGCACTTCCAA
132	cDN12P0001M04	TTGAGGCTCGTCTGGTCC	TACGAGTCGAAGCCATCGT
133	cDN12P0001M17	TCATGAACCTGGAGGCTGT	GGCTGTCATGCGAGTTACAT
134	cDN12P0002A09	TGGGTAAGGTGCCCACTAT	GAAGTCTCCGTCATTGCC
135	cDN12P0002I21	TGCTGCCTACTGTCAGTTCAT	CTTGTCCACATTCATTCTCTTG
136	cDN12P0002N05	CAGTCGGTCACCATCAAGAC	ATCTCCTTCATCGTCCATCC
137	cDN12P0004K24	CTACGACTGCGTCTGCAAGT	CCTCGCACTTATTCCTGTCC
138	cDN12P0004M08	ACCTGTACATCGTAGCGTGC	CACCAGGTGTCCTCAGACAA
139	cDN12P0004O17	ACAGTTCGCCACCTGTACC	TTCTCAATCATCTTGAGCAGC
140	cDN12P0004P11	TCCTTCATGTTCAAGCTCCA	TTCGAACAGGCTGCATAAGA
141	cDN12P0005E13	AATGGCCTCCACCTTCATC	CCAGGTCGTTAAGGTTGCTC
142	cDN12P0005I06	CAGATCACCGCAGATAAGCA	CCTTGAAGGCGAAGTTGAG
143	cDN12P0005I22	GGTGAAACACATCGGCTC	TTAACTTCGTCCACGGCT
144	cDN12P0006G10	CGCTCAGGAGAGACTGGC	GCATCTCCTGGATCTCCATC
145	cDN12P0006J21	CAATAACCTTATCGCCGAAG	ATCCAGCCAACAGAGTCTCA
146	cDN12P0006M10	CTGCTTGAGCAGGCTGAG	AATGAGCGGTTCTGTGTTG
147	cDN13P0001J14	GAAGGCTGCATTGGACAGA	AGCATGAAGCCAAGGAAGA
148	cDN13P0001P21	CCTGCAAGGAACTGAAGGAC	AGGTCCTCAACTGCAGAATCA
149	cDN13P0002N10	GTTGTCGGATCTACCTGCG	CACGAGCTCCTTCAGCTTG

150	cDN13P0003B12	GAATCTGCATGTACTGGCTTG	TGACATCAGGCAGATGGAAC
151	cDN13P0003B14	CCATGCAGAACCTGAACG	GGCCTGGTACTTGCTGTAGTCT
152	cDN13P0004B08	CAGGAACTGCTGGTCCAA	TTCTGGCAGCGGTGATAGT
153	cDN13P0004P17	TGGTTGGCATTACTGTTGGA	GCAGGATCAGCTGGATGAG
154	cDN13P0005B20	CAAGAAGACTGTGGACCTGG	CCGTCAGCCGTTATGATGT
155	cDN13P0005C21	GGCAGTTCGTCAAAGACTCC	AAACCGATGAAACCCATGAT
156	cDN13P0005D10	CCAGCTGAACAAGGACAAGA	CTGTAAGAACGGCAGACGC
157	cDN13P0005H08	GGAGTCCAGAGAGCTACTTCC	CAGAGGAGATGTCTGCGAT
158	cDN13P0005I11	AGATGAACCACAAGATGCTAGA	GTGAGGACGTGACGAAGC
159	cDN13P0005K05	GCAACTGTCATCGTCATTACCT	GGTTCTCATAGCCGTTCTGC
160	cDN13P0006G21	CATCCTGAGTGGCTGGATT	TCTTCGGTACGGTTCATGTC
161	cDN13P0006M16	AAGGCTATGCAGACAAGAACCT	ACTTCCAGCTTGCTCTTGG
162	cDN14P0002B10	TCCATCAACTTCCTGGATCA	GCTCAACTCGCATCGACA
163	cDN14P0002G12	TGGCCTCTGTAATGCAAGAGT	CCATCCAGCTATCAGCAATG
164	cDN14P0004B03	TTCTGTTCGAGTGTTCGCC	TGCAGCCATCAACCTTGTA
165	cDN14P0004J06	TGGAGCTGCTGGACAATACT	AACATGTCGGAAGATTCGATG
166	cDN14P0005N21	CAGGACGCTGTGGAGTGT	TGGTCTCATGAGTCACGTAGC
167	cDN14P0006E20	TTCCAGATCTTGGCGAGTG	AAGGCGAGGCAGGATTCTA
168	SAPD01177|FUGU|4|	GGTTTGAAACGGATGCTG	GAATTGCATTCCTCTTTCCC
169	SAPD01199	GGGTTTCACCTCAACAACCA	ATGCCCTCTAGCCTCACAAA
170	SAPD01507	TCGGAGCAAATTTACAACCC	TCACGTACAAAAATACAGTAGAAAA
171	SAPD01628	GACACCAGCGAGGTCAAACT	GAGGAGGTTAAAACCCTCGG
172	SAPD01628|FUGU|1|	TGTACGAGTGCGAGAAGCA	CCTCGCTAGTCTCCACGTCT
173	SAPD01676|TETRA|4|	AGGCCAGAGTGAGGAGGA	CTTGCTGTGTAGCTGCCTTG
174	SAPD01718	CAACATCCACCCGTAAGGAC	AAGGGTTCCTCCTTCTTCCA
175	SAPD01943	CAAGAACTGGGGAGAGGGAT	GCGGTAGCTAGACATCACCC
176	SAPD01943|FUGU|3|	TCGTTGACAACCACGACAA	CCAGCGGTAGCTAGACATCA
177	SAPD02051	CAAGTCAAAGAGATGCACAATCA	TCCACTCCCCAGACTGTAGG
178	SAPD02089	GAAATGGACCATGATGACGA	GCTGTCTGCCATAGTCTGTCAC
179	SAPD02100	GAACTCATCCAGAAGGGCAA	GCCAGTGATGGTCTCCAAGT
180	SAPD02108	CCAGAAGCAGCTCACAATGA	TGTACTTGGGGTGAAGTGGAG
181	SAPD02110	TTTCTGGATAGAAGTGGACATTTG	CTGTGGTTGATGGCATTCTG
182	SAPD02114	TCCTTTAAGAAACGGGTAAATTG	TTTGAGCATGTACAGAAAACCA
183	SAPD02115	AGGCCTGCTGTGTTGTGAAT	TTTACTTTCGATGGTTATGAGAAA
184	SAPD02117	GAACATGTGGTGTGGTCAGG	GGTTGAAACCGTCTACCGTC
185	SAPD02120	CATCCATCACTGGAGCTGAA	AGCACGAGTGTCACTGCAAC
186	SAPD02144	CCTCACGAAGAAGTACCTGAAGA	GGCTGATCTGGAAGTAACGC
187	SAPD02161	TGTGCTAGTGGGTCTTGACG	ATCCATCACGAAAGGTGGAG
188	SAPD02165	GACACTGGTCGTTACGGCTT	CTGAGAGATTGGAAGGGCTG
189	SAPD02184	GAAGTTGCAGAGGATGTGAAGA	AATGTCTCCTACGAGTGCCTG
190	SAPD02186	TCCGAACTTGGCTGAGATTT	TTAGGGCGAAGAAGAAGGGT
191	SAPD02190	ATACCCATCTTGGGCATGAA	TGATTGTGATCAATCGCCTG
192	SAPD02202	ATTCCTTCATGGGTCAGGTG	TCAGATGTTGGTAAGCACCG

WP11: QTL analysis and framework for QTL exploitation in seabass, seabream, rainbow trout and oyster

Start date or starting event			1			
Activity type [1]			RTD/Innovation			
Participants	CO01	CO01.1	CR05	CR12	CR13	CR15
Person Months	1.4	15.8	6	2.8	2	28.88

Objectives

1. Finalise experimental designs for QTL analysis in the four species.
2. Provide secure database for pedigree, marker and trait data for QTL analysis.
3. Analysis of QTL data from four species.
4. Analysis of eQTL data from trout.
5. Explore the impact of identified QTL with effects on resistance to infectious disease.
6. Outline operational genetic protocols incorporating identified QTL and traditional breeding approaches.

Progress towards objectives

1. Finalise experimental designs for QTL analysis in the four species

Study of the optimum designs for QTL detection in the four species under study were reported in the last annual report. These studies have since been reported at the Genetics in Aquaculture meeting in Montpellier in June 2006 and have been submitted as a peer-reviewed publication. The results of these studies feed into the actual experiments performed in work packages WP05, WP06 and WP07 and the major activity in this area over the last year has been the modification of the proposed designs to deal with the experimental practicalities as they occur. Experimental developments are detailed in the reports on WP05, WP06 and WP07, and briefly outlined below.

WP05 – Oysters. The major summer mortality event was confined to three families rather than five as planned. We advised that selective genotyping of animals from theses three families could provide a powerful design to identify QTL associated with resistance/susceptibility. Additional sampling from one family with a second late round of mortality may identify whether early and late mortality are under similar genetic control. Genotyping is underway to generate the data for QTL analysis.

WP06 – Sea Bream. Problems in setting up challenge studies to obtain approximately equal proportions of survival and mortality have meant that focus has shifted to time between challenge and death. We contributed to the way forward on this study, on the appropriate strategy for determining the level of genetic contribution to this trait and confirmed that the design is appropriate for a QTL study if the trait proves to be heritable. Data collection continues.

2. Provide secure database for pedigree, marker and trait data for QTL analysis

The resspecies database (http://www.resspecies.org) hosted at Roslin has been configured to store the relevant QTL mapping data for sea bream, sea bass and oysters. It has been agreed that the MAPGENA database developed by INRA will be utilized for the data from the trout study. These databases provide secure storage of data but is web accessible to the partners (via a username/password system) to view and access data. The database system provides tools to export the data in various formats suitable for linkage and QTL analysis.

The internal structure of the Resspecies database can only be set up once the pedigrees for the populations have been defined and the phenotypic data finalised. This is currently underway and the major activity of populating the database with data will occur in the next year of the project. In the meantime, entry of marker genotype data for linkage mapping in WP09 continues to the ResSpecies database via the web, with partners 12 and 13 submitting the data as they are generated in the laboratory. Data are screened upon entry and those containing Mendelian errors are flagged and rejected by the database. This allows submitting scientists to rapidly identify errors in the data and correct these before final submission of the data.

3. Analysis of QTL data from four species.

Work on this objective awaits the generation of data in work packages WP05, WP06 and WP07.

4. Analysis of eQTL data from trout.

As indicated below, this objective has become redundant.

5. Explore the impact of identified QTL with effects on resistance to infectious disease.

As planned work on this objective will be initiated when relevant QTL are identified in objective3.

6. Outline operational genetic protocols incorporating identified QTL and traditional breeding approaches.

As planned work on this objective will be initiated when relevant QTL are identified in objective 3.

Deviations from the project workprogram

The current projected work programme follows the original plan with the exception of objective 4. For reasons of cost and time, it is now proposed that eQTL data will not be collected within this project and thus objective 4 concerning the analyses of these data becomes redundant although it is intended that this component of the work will be completed outside the current project.

List of deliverables

Code	Title	Due date	Actual/foreseen achievement date
D11.01	Experimental designs for QTL analysis	12	12
D11.02	Databases to store QTL data for the four species	24	24
D11.03	Identification of loci and QTL that affect stress response or disease resistance in the four species	39	39
D11.04	Linkage analysis between genetic markers and gene expression data in trout	36	Redundant
D11.05	Evaluation of genetic epidemiology of disease resistance QTL	42	42
D11.06	Outline operational genetic protocols for alternative breeding schemes incorporating MAS	42	42

List of milestones

Code	Title	Due date	Actual/foreseen achievement date
M09.01	QTL studies finalised	12	12
M09.02	Database ready to accept QTL data	24	24
M09.03	Pedigree and trait data loaded into database	30	30
M09.04	Expression trait data loaded into database	36	36
M09.05	Marker data loaded into data base	36	36
M09.06	QTL analyses completed	39	39
M09.07	QTL data evaluated and breeding programmes outlined	42	42

Section 3 – Consortium management

Consortium Management tasks and their achievement.

After two years of functionning, the project is now in a cruise mode for management and communication/exchanges between partners: Sub-groups of participants having common objectives and tasks in the project gathered themselves when necessary (mainly through mail exchanges), hopefully without the need for the coordinator to step in. Most of the time of the project meetings is devoted to scientific discussions.

One interesting question raised during the first year of the project was the association with the Network of Excellence Marine genomic Europe who is developping very complementary genomic tools (EST collections spotted on microarrays) as Aquafirst in sea bream, sea bass and oyster. During the second yearn this link has been developped and allowed both projects to gather their efforts and resources: We have fused our EST database, we are constructing a unique microarray bearing EST produced by the 2 projects for seabream, seabass and oyster and we are using MGE genomic resource centre facilities (MPI in Berlin) for spotting these arrays. Finally, development of genomic resources for seabass and seabream has also largely benefited from these close links between the 2 projects.

Finally, the major concern of the coordination is now the delay for delivery of the functional genomic data (part 1 of the project). A new planning has been set up: We have to follow it very closely to be sure that this will not have any impact on the genetic part of the project which averall is in time with the initial plan.

Annex – Plan for using and disseminating the knowledge

Section 1 - Exploitable knowledge and its use

Not relevant to the project

Section 2 - Dissemination of knowledge

Planned/ actual dates	type	Type of audience	Partner responsible /involved
November 2006 (Bruxelles)	'Research on fish farming technologies for SMEs: Technological challenges and market opprotunities' Workshop organized by the European Commission DG Research	Research + Industry	COO1
February 2006 (Faro)	Presentation of the Aquafirst project ate the AQUAFUNC project meeting	Research	CO01

Section 3 – Publishable results

Not yet relevant to the project.