Selective breeding for stress tolerance in aquacultured fish

OBJECTIVES

The overall aim of this study is to assess the feasibility of increasing the tolerance of finfish to stress selective breeding. Because ideal husbandry practices are of necessity compromised by the economic realities of large-scale fish production, stress is an unavoidable component of the finfish aquaculture environment. Although the physiological response of fish to isolated stressors is essentially adaptive, fish under intensive culture conditions are exposed to repeated acute stress and in some instances to chronic stress. In these cases the response becomes maladaptive resulting in adverse effects in growth, flesh quality, reproductive function and immunocompetence (Pickering, 1993; Campbell et al., 1994; Pickering and Pottinger, 1989; Sunyer et al., 1995). Therefore, an enhanced tolerance of stressful procedures is likely to improve food conversion and growth, reduce disease incidence and improve broodstock performance. Thus, an investigation on selective breeding of fish for stress tolerance has been undertaken in the gilthead sea bream, Sparus aurata, and rainbow trout, Oncorhynchus mykiss. The selection procedure is based on traits which are strongly linked with responsiveness to stress, i.e. plasma cortisol and lysozyme, as previous work from the laboratories participating in this work estimated the potential heritability of these traits (Fevolden and Røed, 1993; Fevolden et al., 1991, 1994; Røed et al., 1993a, b; Pottinger and Pickering, 1997).

Sea bream and trout were selected for high or low stress response, measured by plasma cortisol levels, and tested after repeated confinement stress. The consistency of the response and the correlation with other indicators was analysed. After this, crosses between either high cortisol (HR) male and female responders and low cortisol (LR) responders were made. Reproductive parameters were measured and correlated with cortisol. The results show that selected fish consistently show the corresponding high or low response and that growth rate is higher in HR. Reproductive parameters show slight differences in trout between HR and LR whereas in sea bream, HR show lower egg quantity but better quality. F1 generation shows the same pattern of cortisol responsibility as the respective progenitors.

MATERIALS AND METHODS

Sea bream

Broodstock of sea bream of 900 g (147 fish) from both Mediterranean and Atlantic populations were subjected to three hours’ confinement in their own tanks by restricting the living space with net walls. In this way, space restriction is achieved, but avoids loss of water quality, as water flow and renovation are
always maintained. After confinement, fish were individually netted, anaesthetised and weighed, and blood was sampled through caudal puncture. This procedure was repeated every month during five consecutive months (June to October). Plasma and serum were obtained after centrifugation and concentrations of cortisol, glucose, and lactate, and osmolality and agglutination activity were determined. Once the fish samples were analysed for cortisol response to the stress, two males and one female of fish of high cortisol response and the same for low cortisol response were placed in individual tanks. The fish were classified as high cortisol responders (HR) or low cortisol responders (LR) depending on the consistency of the response to the stress during the five months. The offspring of these families were analysed in terms of quantity and quality of the eggs. The variables measured were number of eggs per female, spawning viability, percentage of hatched eggs and larval size after hatching.

**Trout**

Two-year-old mixed sex rainbow trout were individually identified and subjected to three hours’ confinement (10 fish per 50 l tank) at monthly intervals. After confinement fish were anaesthetised and 1 ml of blood was removed. Plasma cortisol, glucose and lysozyme levels were determined. To perform the crosses, 15 females from the HR and LR groups were mated with the corresponding 15 males. In addition, 15 males and females randomly chosen from the US group (unselected fish) were mated. The total egg volume and egg size, egg mortality and sperm count were determined.

**RESULTS AND DISCUSSION**

The results of the selection procedure in terms of the cortisol response have resulted in a consistency, as the fish selected as either high or low response continued showing high and low levels respectively in sea bream and trout. Similarly, in both species a positive correlation was found between growth and high cortisol response (see Table 1). As regards the differences between species, the following results were obtained:

<table>
<thead>
<tr>
<th></th>
<th>Cortisol (ng/ml)</th>
<th>Growth rate (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sea bream</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High responders</td>
<td>109.1 ± 21.1</td>
<td>3.13 ± 0.48</td>
</tr>
<tr>
<td>Low responders</td>
<td>60.6 ± 12.2</td>
<td>2.47 ± 0.34</td>
</tr>
<tr>
<td><strong>Trout</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High responders</td>
<td>110.8 ± 8.9</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>Low responders</td>
<td>42.9 ± 3.4</td>
<td>0.76 ± 0.04</td>
</tr>
</tbody>
</table>

**Sea bream**

The results obtained up to now regarding reproductive parameters show the following trends: only 65 % of the pairs selected for HR or LR spawned whereas 100 % of unselected pairs spawned. No correlation was found between the number of spawns and post-stress plasma cortisol in females. There were no significant differences in the number of eggs per kilogram of female between HR and LR. However, both groups had significantly more eggs per kilogram of female per day than the US group. Egg quality in terms of viability and hatching rates was significantly better for HR than the other groups, whereas lipid droplet diameter was lowest in the US group. No significant differences were found for newly hatched larvae length between groups (see Table 2).
5.5. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.1. Genetics

**Trout**

The evaluation of the impact of a stressor on the reproductive system of selected fish showed that confinement caused a clear decrease of 17-B oestradiol but no differences between HR and LR. No significant differences were found in the gonadosomatic index (GSI), number of eggs per female, individual egg weight, sperm counts in males or egg mortality at hatching and at eyeing. However, egg volume was lower in LR and sperm count was higher in HR and LR than in unselected fish (US). See Table 3.

The first examination of the stress responsiveness of the F1 strongly indicates that the fish display the same traits for HR or LR as the parent generation. Up to three assessments on the progeny show high significant differences in post-stress cortisol levels in HR than in LR.

Different results have been found as regards the metabolic response of the progeny of HR or LR fish under chronic stress. Unstressed fish show a better growth rate and feed efficiency in the LR group, whereas in fish subjected to high density, the growth rate and feed efficiency were significantly lower in HR and the condition factor was lower for the HR crowded fish. Liver alanine aminotransferase showed lower activity in LR both in crowded and uncrowded fish and pyruvate kinase showed higher activity in HR in both groups. Phosphofructokinase increased in the HR crowded group.

Regarding heritabilities of selected traits such as cortisol and lysozyme, the results showed moderate to high heritability estimates, whereas the common full-sib effects due to effects other than additive effects were negligible.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR</th>
<th>LR</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of spawns</td>
<td>41.37 ± 32.33</td>
<td>26.74 ± 28.52</td>
<td>41.80 ± 25.06</td>
</tr>
<tr>
<td>Eggs/kg of female/day</td>
<td>51 807 ± 16 492</td>
<td>54 243 ± 26 825</td>
<td>33 172 ± 16 491</td>
</tr>
<tr>
<td>Egg viability (%)</td>
<td>61.24 ± 28.63</td>
<td>42.82 ± 27.37</td>
<td>54.74 ± 26.69</td>
</tr>
<tr>
<td>Hatching rate (%)</td>
<td>84.83 ± 21.61</td>
<td>74.88 ± 33.96</td>
<td>77.43 ± 27.32</td>
</tr>
<tr>
<td>Egg diameter (mm)</td>
<td>0.957 ± 0.016</td>
<td>0.966 ± 0.018</td>
<td>0.964 ± 0.015</td>
</tr>
<tr>
<td>Lipid droplet diameter (mm)</td>
<td>109.1 ± 21.1</td>
<td>93.78 ± 14.50</td>
<td>109.1 ± 21.1</td>
</tr>
<tr>
<td>Newly hatched larvae length (mm)</td>
<td>60.6 ± 12.2</td>
<td>74.13 ± 10.16</td>
<td>60.6 ± 12.2</td>
</tr>
</tbody>
</table>

HR = high responders; LR = low responders; C = control (unselected group); SD = standard deviation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR</th>
<th>LR</th>
<th>US</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-ß oestradiol (ng/ml) (1)</td>
<td>24 ± 2</td>
<td>19.9 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>Gonadosomatic index</td>
<td>14.4 ± 0.6</td>
<td>15.5 ± 0.9</td>
<td>14.3 ± 0.4</td>
</tr>
<tr>
<td>Number of eggs/female</td>
<td>2 776 ± 179</td>
<td>2 541 ± 165</td>
<td>2 385 ± 89</td>
</tr>
<tr>
<td>Individual egg weight (mg)</td>
<td>80.2 ± 8.5</td>
<td>75.3 ± 2.2</td>
<td>78.4 ± 1.5</td>
</tr>
<tr>
<td>Egg volume (mm³)</td>
<td>80.8 ± 1.8</td>
<td>74.3 ± 2</td>
<td>79.3 ± 3.1</td>
</tr>
<tr>
<td>Egg mortality at hatching (%)</td>
<td>19 ± 2</td>
<td>15 ± 5</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>Egg mortality at eyeing (%)</td>
<td>8 ± 1</td>
<td>5.3 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Sperm count (x 10³ per ul)</td>
<td>13 432 ± 1 522</td>
<td>14 786 ± 1 302</td>
<td>9 577 ± 1 143</td>
</tr>
</tbody>
</table>

(1) The oestradiol measurement is after 48 hours’ confinement (maximal suppression).
In conclusion, these preliminary results show that in sea bream and trout the selection for cortisol appears to be consistent and heritable. Nevertheless, it is not clear whether selection for low cortisol response is always recommendable. Further analysis will be carried out on the consistency of stress/cortisol response on this and the next generation to see whether the selection for this characteristic may help to decrease husbandry problems and improve the profitability of aquacultured fish.

REFERENCES


Fevolden, S. E., Refstie, T. and Røed, K. H. (1991), ‘Selection for high and low cortisol stress response in Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss)’, Aquaculture, 95, pp. 53–65.


### Table 4: Metabolic parameters in trout progeny subjected to chronic stress (mean ± SE(M))

<table>
<thead>
<tr>
<th></th>
<th>SGR</th>
<th>FE</th>
<th>CF</th>
<th>AAT</th>
<th>PK</th>
<th>PFK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncrowded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LR</td>
<td>2.92 ± 0.02</td>
<td>1.24 ± 0.03</td>
<td>16.3 ± 0.18</td>
<td>183.8 ± 2.21</td>
<td>110.7 ± 4.8</td>
<td>36.9 ± 1.8</td>
</tr>
<tr>
<td>HR</td>
<td>2.26 ± 0.07</td>
<td>1.10 ± 0.01</td>
<td>15.0 ± 0.12</td>
<td>246.6 ± 22.1</td>
<td>139.8 ± 6.1</td>
<td>33.9 ± 0.3</td>
</tr>
<tr>
<td>Crowded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LR</td>
<td>1.96 ± 0.05</td>
<td>1.21 ± 0.02</td>
<td>14.83 ± 0.44</td>
<td>269.6 ± 16.2</td>
<td>11.9 ± 1.8</td>
<td>40.12 ± 2.1</td>
</tr>
<tr>
<td>HR</td>
<td>1.66 ± 0.03</td>
<td>1.13 ± 0.01</td>
<td>12.73 ± 0.60</td>
<td>260.4 ± 20.6</td>
<td>131.2 ± 0.9</td>
<td>40.62 ± 1.3</td>
</tr>
</tbody>
</table>

LR = low responders; HR = high responders; SGR = specific growth rate; FE = feed efficiency; CF = condition factor; AAT = alanine aminotransferase; PK = piruvate kinase; PFK = phosphofructokinase

5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.1. Genetics

FAIR-CT95-0421

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

OBJECTIVES

The EU project Genephys, initiated by the Ifremer laboratory GAP (Génétique Aquaculture et Pathologie) at La Tremblade, and CREMA (Centre de Recherche en Écologie Marine et Aquaculture) at L’Houmeau, aims to establish the relationships between physiological traits involved in growth (oxygen consumption, absorption efficiency, scope for growth) and their genetic bases (determinism, variability within and among populations) in cupped oyster, Crassostrea gigas.

The main objectives are:

1. to express the maximum variability of growth, at both the larval stage and the adult stage, in an oyster population originating from parents of various origins;
2. to check the persistence over time of growth performance and related physiological functions;
3. to explain the observed growth differential by physiological functions studied;
4. to establish whether the growth differential by physiological functions is associated with a genetic basis, using two types of markers (allozymes and microsatellites), and whether these are related to aneuploidy and the identity of missing chromosomes;
5. to estimate, in a second generation, genetic variance (additivity, dominance) and heritability of growth and of related physiological traits and also to estimate aneuploidy transmission to this generation;
6. to evaluate the possibility of selection for a better assimilation efficiency.

The beginning of a genetic map provides the first step for marker-assisted selection and QTL (quantitative trait loci) location.

DESCRIPTION OF WORK

This study uses experimental populations of the Pacific cupped oyster, Crassostrea gigas, to examine genetic and physiological mechanisms behind growth patterns in this species. To this end, experimental progenies were produced using oysters from different populations to provide a large genetic base for investigation. The crosses were made principally in 1996, the first year of this five-year project. The resulting generation, G1, has been studied in depth for characters of interest and the possible relationships between genetics and physiology. Work on the project has involved the development of new techniques in different fields of biology in order to examine these traits on the animals. Study of G1 occupied much of the second and third years of the project.
and has provided a large amount of data, much pertaining to a common group of individuals on which several different experiments were conducted. We are now in a position of synthesis for the data gained for the G1 generation from work done by the six different partners and nine laboratories involved in the project. The G1 generation was also used to produce a G2 generation, which consists of different progenies which will be used for different investigations. The breeding of a second experimental generation from the first will enable us to gain an idea of inheritance of physiological parameters which are important for productivity in the species. One set of genitors was selected from G1 animals according to physiological performance and a second was chosen for levels of somatic aneuploidy (missing chromosomes). The G2 was successfully produced in 1998 and studies on this generation will commence in 1999.

STATE OF PROGRESS

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

Production and management of the G2 generation

This task was performed in 1998 using animals scored for the stability and efficiency of physiological performance in 1997–98 and populations displaying different levels of somatic aneuploidy. The first part of the G2 generation is presently being raised under controlled conditions in preparation for growth and physiological study in the coming two reporting periods. It consists of progenies of crosses between combinations of parents of high and low performance. Four progenies have already been selected for study and individual size measurements begun. G2 animals for the study of inheritance of aneuploidy were bred using parents from populations of contrasting aneuploidy level. Four progenies available for study issue from crosses between and within groups of high and low aneuploidy.

Recording of growth performances

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

Physiological analysis

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

The development of new continuous or sequential measurement systems for respiration and filtration have allowed animal activity to be quantified over periods of 24 hours.
The physiological performances of laboratory-raised animals were shown to be stable over time (based on five experiments over the course of a production cycle of eight months). This result shows that in production conditions where food is not a limiting factor for growth, animals which perform best at one period of the year also perform best during other seasons.

Parents for the second generation were ranked according to an index incorporating the activity and intensity of physiological functions concerning the acquisition and use of energy. The animals with the highest performance have greater absorption and lesser oxygen consumption than those with the lowest performance. For the first time in this study a linear relationship between oxygen consumption and animal dry weight was observed (p < 0.01).

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

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

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

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

**Genetic analysis**

The allozyme and microsatellite analyses have been completed on G1. Both methods show that there is an imbalance in parental contribution in the progeny. The results of the microsatellite analysis performed on larvae and juveniles from one set of G1-controlled crosses show that parental contribution is unbalanced from the earliest stages of life, that it changes over time (a feature already exposed in the allozyme study) and that gametic competition occurs during fertilisation. Results of the microsatellite study of G1 adults emphasised that only a limited number of parents used in the crosses made for G1 may be represented in the progeny and therefore that genetic variation is less than the potential suggested by the parent used in a crossing design. Parental effects on growth in the growth experiment in Bouin will be analysed in the coming reporting period.

Allozyme techniques were applied to the study of heterozygotes in G1 this year. Heterozygote deficiency did not appear to differ consistently with age class, i.e. despite indications from microsatellites that overall variability is reduced, this does not appear to be manifested as a reduction in heterozygotes with increasing age of the experimental population. Deficit of heterozygotes did not appear to be linked with growth slope either but did appear at greater levels for some loci in the progeny of some intra-population crosses more than others.
Aneuploidy study has advanced with the completion of banding maps for the identification of missing chromosomes in aneuploid cells. Aneuploidy itself has been examined in animals of different size from contrasting populations and once again has shown that there is a negative relationship between aneuploidy and size and that there is a population (genetic) effect. In the coming reporting period, the untangling of size and genetic effects will be examined using samples of the same size from a series of full-sib families treated for chromosome study this year. The inheritance of aneuploidy will be examined in G2 families issued from crosses made this year between the populations with different levels of this trait.

**ACHIEVEMENTS**

The experimental studies on the G1 have now been completed.

This year these have yielded:

- results from the growth experiment at Thau indicating that the influence of initial size is environment dependent and that small oysters can achieve higher specific growth than large ones;
- the confirmation of stability of physiological traits (oxygen consumption and filtration) over a period of eight months during which the same individuals were measured repeatedly. Different individuals showed different performances and degrees of stability but overall statistics showed animals to be stable;
- the demonstration that faster whole-body protein synthesis in slower growers stems from fundamental metabolic differences in non-digestive tissues, and is not directly related to feeding rate;
- the support for enzymatic results distinguishing different sub-groups of an experimental sample (digestive enzymes activities, digestive enzyme Michaelis constants) by genetic differences;
- the application of allozymes and microsatellites showing that parental contribution is unbalanced and changes over time;
- the scoring of aneuploidy in populations of different origin and animals of different size confirming the negative correlation of the trait with size and providing further evidence for a genetic basis;
- the creation of G2 progenies for study of physiological traits and aneuploidy;
- the analysis data by each partner individually.

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

**FUTURE ACTIONS**

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

- study of individual growth performances;
- physiological performances (non-destructive study of filtration and oxygen consumption);
- protein turnover and proteolytic activity;
- digestive enzyme activities;
- inheritance of aneuploidy.

Work will continue to integrate the G1 data from the different experiments completed in the preceding reporting periods. This should provide important insight into the genetics and physiology of growth in *Crassostrea gigas*.
**5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE**

5.3.1. Genetics

**FAIR-CT96-1591**

*Generation of highly informative DNA markers and genetic marker maps of salmonid fishes — Salmap*

**OBJECTIVES**

1. Production and characterisation of highly informative DNA markers with particular stress on microsatellites in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*).
2. Run characterised markers through reference families, some of which are also typed for allozymes, to provide recombination data for linkage analysis. Typing of traditional diploid pedigrees will allow for sex linkage analysis.
3. Carry out genetic linkage analysis and construct species-specific low-resolution genetic maps, embracing mostly anonymous markers but also enzyme loci (rainbow trout).
4. Depending on the success rate of cross-reacting markers and the degree of interspecific conservation of marker linkage groups, identify anchor loci in a comparative salmonid map.

**DESCRIPTION OF WORK**

The Salmap consortium will isolate and characterise highly informative DNA markers using standard molecular genetics methods (e.g. screening genomic libraries for microsatellite positive clones, DNA sequencing, PCR analysis) from three species of salmonid fish: Atlantic salmon, rainbow trout and brown trout. Major efforts will be made on the isolation, sequencing and characterisation of microsatellite markers, predominantly of the dinucleotide repeat type. Tri- and tetranucleotide repeat microsatellites will also be isolated to some extent. Segregation studies of these markers in appropriate reference families will be carried out using PCR and electrophoretic techniques. Subsequent linkage analysis of the segregation data, using contemporary and consensus computer programs, will enable the construction of species-specific linkage maps and possible salmonid comparative maps.

**STATE OF PROGRESS**

At the end of the first reporting period of the project, the state of progress was as follows:

*Atlantic salmon*: 278 (256 from Atlantic salmon + 22 from rainbow trout) microsatellite loci have been identified and partially characterised and so far 55 (38 from Atlantic salmon + 17 from rainbow trout) have been fully charac-
5.3.1. Genetics

Rainbow trout: 172 (155 from rainbow trout + 17 from Atlantic salmon) microsatellite loci have been identified and 124 (116 from rainbow trout + 8 from Atlantic salmon) have been fully characterised. In all, 108 microsatellite loci and six RAPDs have been genotyped in reference families resulting in the identification of 20 linkage groups by the Canadian group and two linkage groups were identified by the French group.

Brown trout: 89 microsatellite loci (50 from rainbow trout + 39 from Atlantic salmon) have been identified and 63 microsatellite loci have been fully characterised (41 from rainbow trout + 22 from Atlantic salmon). In all, 15 have been genotyped in reference families resulting in the identification of three linkage groups.

ACHIEVEMENTS

Within the first 12 months of this project, 430 microsatellite PCR assays have been described for salmonids — this represents one of the largest databases of this kind generated to date. In isolating these microsatellites, several experimental approaches have been tested. Screening of a range of fragment lengths has identified an optimal insert length of 300–500 bp for PCR-amenable microsatellite typing. Microsatellite library enrichment protocols have been successfully employed for several of the libraries developed. Finally, the first microsatellite-based linkage groups have been described for rainbow and brown trout.

FUTURE ACTIONS

Research will continue towards the genetic mapping of the three salmonid species as outlined in the work programme. Work will concentrate on describing the first microsatellite-based linkage groups in Atlantic salmon, exploiting markers generated within the project as well as those published elsewhere. In a similar manner, the existing linkage groups for brown and rainbow trout will be further developed. Successful completion of this work will result in low-resolution genome maps suitable for future QTL exploitation and broodstock management.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.1. Genetics

FAIR-CT96-1981

Development of sustainable aquaculture of Arctic charr

OBJECTIVES

This text briefly describes the basic objectives and initial achievements of the project Aquacharr. Work is in progress and no direct results are presented.

The project focuses on the development of aquaculture of the salmonid fish Arctic charr, Salvelinus alpinus. Aquaculture of Arctic charr is in its initial stages but is clearly growing in Europe. This trend is also apparent in Canada. In order to ensure profitability and competitiveness, this new industry is in great need of organised research and developmental work.

The Aquacharr project is young. It started in December 1996 and no direct results can be published at this stage. In this paper, the main objectives of the project, a description of the work and an overview of the present state of progress and achievements are given.

In addition to the partners listed, parts of the work are subcontracted to the Icelandic Marine Research Institute, the Icelandic Agricultural Research Institute, and the aquaculture breeding company Stofnfiskur Ltd.

The primary objective of the project is to provide a sound, multidisciplinary scientific and technological basis for the controlled development of an environmentally friendly and competitive aquaculture of the salmonid fish Arctic charr, Salvelinus alpinus, in Europe. To do this we aim to:

• develop and test microsatellite probes as a tool to combat the pressing problem of genetic mixing of cultured and wild stocks;
• find molecular markers for economically important traits to improve the efficiency of breeding programmes for this species;
• identify and quantify biological variability in economically important traits and their genetic and environmental origin.

The project is divided into two main tasks, which are divided into several sub-tasks.

Objective of task A

The objective of task A is to develop and enhance the application of techniques for genetically characterising domesticated stocks of Arctic charr as well as wild populations in areas of Europe of high potential risk of genetic mixing and/or of high conservation or economic value. This is essential in the development and implementation of European regulations concerning confinement of domesticated stocks.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.1. Genetics

Task A is divided into two sub-tasks. In sub-task A1 microsatellite primers are developed for the individual stock identification of Arctic charr. In sub-task A2 selected wild and domestic populations of Arctic charr in Iceland, Scotland, Ireland and Sweden are sampled and genetically analysed based on the findings in sub-task A1. This sampling also involved documentation on basic phenotypic characteristics of the charr as well as other biological, chemical and physical properties of their environment.

Objective of task B

The objective of task B is to develop a dynamic strategy for the genetic improvement of Arctic charr used in European aquaculture using traditional quantitative genetics as well as the latest developmental and molecular techniques. A European breeding plan for Arctic charr will be formulated using information available from breeding programmes in Sweden and Iceland, together with results emerging from the current project.

Task B is divided into five sub-tasks. In sub-task B1 available data from Arctic charr breeding programmes in Iceland and Sweden will be analysed for the potential effects of major genes on performance characteristics. In sub-task B2 genetic probes (PCR) are designed to detect single gene polymorphism in genes potentially related to performance in aquaculture strains of Arctic charr. In sub-task B3 long-term rearing experiments are conducted on families and stocks of Arctic charr to examine genetic and environmental interactions. In these experiments charr are reared under different environmental conditions in order to determine if families and stocks respond differently to varying rearing conditions. In sub-task B4 rearing experiments are conducted to correlate embryonic performance features, primarily variable metabolic rates, to later growth and performance in culture. Specifically, what kinds of genes are expressed in relation to this performance variation are determined. In sub-task B5, which will take place in the final year of the project, all the available information will be synthesised and an overall proposal for the development and breeding of cultured Arctic charr in Europe will be formulated.

RESULTS AND DISCUSSION

In sub-task A1 five microsatellite primers have been developed that provide good resolution in detecting stock-specific genetic variation. This is a significant milestone for the project. In sub-task A2 genetic samples have been provided from a number of wild Arctic charr populations in Iceland and Scotland. Biological and physical data were obtained in the respective freshwater systems.

In sub-task B1 data from Iceland and Sweden have been organised for future analysis. In sub-task B2 significant variation in performance genes has been detected in two Icelandic and one Swedish aquaculture stocks of Arctic charr. In sub-task B3 gene environment rearing experiments are ongoing in Iceland and Sweden involving several strains of Arctic charr. The environment in these experiments differs primarily with regard to temperature. Performance parameters such as growth patterns, sexual maturity, oxygen consumption and food intake are estimated regularly. In sub-task B4 experiments have been ongoing linking variation in early development performance, mainly metabolic rate, with later performance. Studies to identify differential gene expression in high- and low-metabolic-rate charr are ongoing and have already produced preliminary results.

In general, the project is progressing as planned. The main achievement of the project at this stage is the development of microsatellite primers. Secondly, the detection of polymorphism in performance genes in sub-task B2 is of great importance for the future development of breeding techniques for this species. The experimental work in sub-task B4 has produced very important information about variation in early development and quantified metabolic rate difference which are being examined genetically using novel techniques.
**FAIR-CT98-3482**

*Improvement of transgenic technologies in fish: assessments and reduction of risks*

**OBJECTIVES**

This project aims at improving transgenic technologies in fish, through a better control of integration and expression of the introduced genes. Stable integration must occur at regular rates, and progress must be made to integrate the transgenes into a limited number of sites in the genome. Expression must be reproducible, and attain and not overstep the desired levels. To meet these requirements, an important effort will be devoted to the development of new instruments, some of which have already been validated in other organisms, but also to the improvement of the actual transgenic technology used in fish. Particular attention will be given to the control of integration sites in parallel with the achievement of site-independent expression. The routes to improved transgenics will include gene transfer experiments into both the germline, either directly *in vivo* or via cultivated embryonic stem cells and primordial germ cells, and into the soma.

Central to this project is the development of strategies to improve the technology to a point where the introduction of foreign genes into fish becomes a reliable method for systematic alterations of the genome being under the absolute control of the researcher and giving the expected result. How fish genes in general and in detail work is still an unsatisfactorily answered question, which is of major interest for aquaculture, fisheries and all fish-related basic research. The project should develop transgenic technologies that will provide the appropriate tools for answering these questions.

It is not intended within this project to produce genetically modified fish for consumers’ use. The pure research-on-technology oriented nature of the questions addressed experimentally is reflected in the fact that all reporter and indicator genes used are exclusively those which will not have any physiological effect on a cell or a whole organism. No release of genetically modified organisms is intended as part of the scientific programme.

All transgenic research will be performed within guaranteed safe laboratory containment under EU regulation and established national legislation. To cope with possible public concerns, the scientists involved in this project intend to set up the safest conditions for handling transgenic fish and will apply them to their own research even if they exceed the legislation of their own country. They will provide a basis for those countries where no legislation for research on genetically modified fish exists so far.

Techniques for a non-genome-invasive somatic gene transfer are further elaborated in this project, which will prevent the non-reversible situation of stable
transgenesis. Methods will be developed that will allow the selection in vitro of useful transgenic genomes, thus avoiding unnecessary production of transgenic fish in the future.

Inherent to the scientific plan is the improvement and evaluation of methods for monitoring the presence of transgenes in trace amounts in the whole organism, cell and tissue extracts, or any other product derived from a transgenic fish. The most predictable method of transgenic technology, namely the targeting of genes, will be followed up, aiming at the minimal genetic alteration of the modified organism and opening up the possibility to remove unwanted genetic traits or even transgenes once they become undesirable in a certain fish stock of scientific or economic importance.

Following these goals should also enable the scientific community, within a European dimension, to catch up, where necessary, or to follow the enormous progress made in many countries outside the European Community in the field of transgenic research. It should create the necessary expertise to make the optimal use of this novel technology for the sake of basic and applied research as well as aquaculture whenever it becomes appropriate.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION
OF AQUACULTURE

5.3.1. Genetics

FAIR-CT97-3796

Basis of sex determination and gonadal sex differentiation for sex control in aquaculture

OBJECTIVES

This project will investigate the sex determining cascade at the molecular level by means of two complementary approaches.

First, a sex determining factor (SDf) will be searched for in the platyfish, as regions adjacent to the SDf have already been cloned and well characterised in that species. This will be performed through work on the Y chromosome of the platyfish.

Second, differentially expressed genes during gonadal sex differentiation will be searched for using trout and tilapia monosex populations.

Finally, some of the most relevant genes characterised by both approaches will be studied with respect to gonadal sex differentiation in a commercially important aquaculture species in which sex control is of particular importance and not yet achieved, i.e. the sea bass. At the non-molecular level, sex determination will be analysed in turbot: balanced sex ratios are generally observed in turbot, suggesting a simple monofactorial sex determination. However, skewed male proportions could appear in some special cultured conditions, suggesting a possible involvement of other factors. The project plan is to investigate a possible involvement of temperature in turbot sex differentiation by non-molecular approaches. In turbot, male and female breeders resulting from masculinising and feminising treatments are already available. Treatments will be used to analyse the genetic sex determination and potential role of temperature on sex differentiation. Temperature/genotype interactions will be studied in the tilapia model in which genetic sex determination and temperature influence on sex differentiation have already been demonstrated.

The project is an original contribution to sex control in fish. The objectives are threefold:

1. identification of a fish sex determining factor (SDf) by a reverse genetic approach;
2. identification of genes involved in fish sex differentiation;
3. transfer of knowledge in two important aquaculture species: sea bass and turbot.

Sex control is almost always a concern in aquaculture. Sex determination in vertebrates is considered as a switch mechanism that determines the fate
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.1. Genetics

of the undifferentiated gonads through the activation of a cascade of sex determining genes (McElverereavey et al., 1993). In mammals, sex is strictly dependent upon sex chromosomes: inheritance of a Y chromosome results in male differentiation. The sex determining gene, SRY in man, has been cloned and proved to masculinise XX transgenic female embryos in the mouse (Koopman et al., 1991). Some of the genes implicated in the early stages of gonadal differentiation have been shown to be sex linked. This is, for instance, the case for SRY and ZFY in the mouse (Zwingman et al., 1993). Some other genes implicated in this cascade have been shown to be conserved and expressed during sex differentiation in different classes of vertebrates, i.e. Sox9 gene expression in differentiating testis (Kent et al., 1996). In fish, the search for sex-specific probes has been a challenge for many years now and despite many efforts this has been achieved in only a very few species: coho salmon (Forbes et al., 1994), chinook salmon (Devlin et al., 1991), lake trout (Reed et al., 1995) and Leporinus elongatus (Nakayama et al., 1992). All these sequences do not show any homology with known genes involved in the sex determining cascade. All approaches looking at already-known sex-specific sequences in other vertebrates have failed: repeat sequences, human HPRT gene, Bkm satellite, ZFY, SRY, polysat 3TM probe (Tiersch et al., 1992; Ferreiro et al., 1989; the AIR2-CT93-1543 project).

Our project will thus use a completely different strategy by investigating the sex determining cascade by two complementary approaches. First, we will search for a sex determining factor (SDF) in the platyfish which is probably one of the best models for this investigation in non-mammalian vertebrates, as regions adjacent to the SDF have been already cloned and well characterised in that species (Förnzler et al., 1996; Wittbrodt et al., 1989, 1992). This will be performed through work on the Y chromosome of the platyfish. Second, we will search for differentially expressed genes during gonadal sex differentiation. Finally, some of the most relevant genes characterised by both approaches will be studied with respect to gonadal sex differentiation in a commercially important aquaculture species in which sex control is of particular importance and not yet achieved, i.e. the sea bass.

In the sea bass, genetic sex determination has not been understood by classic genetic analyses (the AIR2-CT93-1543 project), and in cultured conditions high male proportions are consistently found (Blazquez et al., 1995). On the contrary, balanced sex ratios are generally observed in turbot, suggesting a simple monofactorial sex determination. However, skewed male proportions could appear in some special cultured conditions, suggesting a possible involvement of other factors. Temperature has already been shown to influence sex differentiation in tilapia (Baroiller et al., 1995). We thus plan to investigate this possible involvement of temperature in turbot sex differentiation by non-molecular approaches. In turbot, male and female breeders resulting from masculinising and feminising treatments are already available. They will be used to analyse the genetic sex determination and potential role of temperature on sex differentiation in turbot. Temperature/genotype interactions will be studied in the tilapia model in which genetic sex determination and temperature influence on sex differentiation have already been demonstrated.

These objectives will be facilitated by the use of suitable animal models and two important commercial species.

The use of suitable animal models

- The platyfish (*Xiphophorus maculatus*) in which the sex determining (SD) gene is in close vicinity with a specific pigmentation locus already cloned (Förnzler et al., 1996; Wittbrodt et al., 1989, 1992).
- A tilapia species (*Oreochromis niloticus*) in which genetic sex determination (XX/XY) and temperature influence on sex differentiation (TSD) have already been demonstrated (Mair et al., 1991).
- The rainbow trout (*Oncorhynchus mykiss*) in which there is a strict monofactorial sex determination (XX/XY) (Johnstone et al., 1979).
In trout and tilapia, gonadal sex differentiation has been described and genetic male and female monosex populations are already available.

The use of two important commercial species
Turbot and sea bass are two important commercial species for European aquaculture in which sex control is of particular importance (females grow better than males) and not yet achieved. They will be the target species of the project.

REFERENCES
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5.3.1. Genetics

**FAIR-CT97-3882**

**Concerted action on identification, management and exploitation of genetic resources in brown trout (Salmo trutta)**

**OBJECTIVES**

**Summary**

The brown trout (*Salmo trutta*) has considerable socioeconomic importance in many European countries. Effective conservation of brown trout stocks requires knowledge of the phenotypic and genetic diversity present in natural populations. Genetic considerations impinge on management of the species in relation to regulation of sustainable fisheries, supplemental stocking of extant populations, and re-establishment of brown trout in areas where the original populations have been extirpated. A knowledge of the genetic resources available within the species is also essential for development of the brown trout for aquaculture. Much high-quality research on brown trout population genetics takes place at the national level within the EU. However, at present there is limited collaboration among research groups in different countries, and little coordination of research priorities. The concerted action aims to improve coordination of the research and provide suggestions for management of genetic resources of the species on a European level. These objectives will be met over the course of two years through an integrated programme of workshops, exchange visits, and questionnaire distribution, and the results of the concerted action will be available through the world wide web.

**Introduction**

The brown trout is a species of tremendous importance in commercial and sport fisheries in several countries of the EU. Thus, it is a species of large socioeconomic importance, especially in less industrialised regions of the EU where angling for brown trout is a cornerstone in maintaining and developing a tourist industry. In addition, research results have demonstrated that there is a large potential for industrial rearing of this species in aquaculture (Krieg et al., 1992). A number of studies have shown that the brown trout exhibits very strong population subdivision (reviewed by Ferguson, 1989). On a large geographical scale, several genetically distinct evolutionary lineages have been identified (e.g. Bernatchez and Osinov, 1995; Giuffra et al., 1994, 1996; García-Marin and Pla, 1996), but also within smaller geographical regions pronounced genetic differentiation may be present (e.g. Ryman et al., 1979; Ferguson and Taggart, 1991; Hansen and Loeschcke, 1996; Apostolidis et al., 1997). Presently, a large number of trout populations must be considered endangered due to environmental degradation and management procedures involving stocking of domesticated trout into wild populations which is likely to compromise the integrity of indigenous gene pools (e.g. Hindar et al., 1991; Largiader and Scholl, 1996). It is necessary to conserve many populations of brown trout, both on a large and
small geographical scale, in order to maintain a wide range of genetic resources available to rearing of the species in aquaculture. Also, populations may be locally adapted, and therefore it may be difficult to establish new populations in areas where indigenous populations have been extirpated. It is evident from the scientific literature that a lot of high-quality research on brown trout population genetics takes place at the national level in many European countries. However, there is little communication among the groups, virtually no coordination of the research, and, while a number of very useful molecular markers have been developed (e.g. Estoup et al., 1993; Prodıhl et al., 1996; Gross and Nilsson, 1995), only little transfer of technology takes place among laboratories.

RESULTS AND DISCUSSION

This concerted action aims to bring together the key laboratories working on population genetics of brown trout in order to coordinate the research, harmonise and, to a certain degree, standardise the use of techniques, and review data and results from the studies that have been undertaken so far. In order to fulfil the management goal of successful conservation of genetic resources and re-establishment of trout populations, it is necessary to obtain knowledge about large-scale variation (phylogeography), small-scale variation (local genetic structure), life history variation (relationship between resident and migratory trout), population history, relationships between ecological and genetic parameters (census population sizes versus effective population sizes), release strategy (choice of stocking material and numbers and types of spawners, genetic impact of stocking and translocations on native gene pools) and harvesting strategy (allowable catch in relation to abundance and size structure). Also, recent technical developments, for instance analysis of DNA markers obtained from archival samples (scale samples — Nielsen et al., 1997), combined with new theoretical approaches (estimation of effective population sizes — Jorde and Ryman, 1996; inference of patterns of gene flow — Tufto et al., 1996) may lead to further new insights. The present state of knowledge on these topics will be reviewed and specific recommendations for a European strategy for management and exploitation of brown trout genetic resources will be given. The synergistic effects of bringing together European scientists with complementary expertise will have a high priority in the concerted action.

The concerted action started on 1 January 1998 and will end on 31 December 1999. The practical framework of the concerted action includes a survey of research activities, collection of published and unpublished results, two workshops involving all participants, and exchange of scientists among laboratories in order to increase technology transfer and perform joint data analyses. Databases on available genetic markers and published and unpublished data will be established, and, together with reports and results of surveys, be made available to other scientists, fishery managers and the aquaculture industry on the world wide web.

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5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE
5.3.1. Genetics


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5.3.1. Genetics

FAIR-CT97-3886

Assessment of procedures for the development of a European standardised multi-site testing programme: application to sea bass, Dicentrarchus labrax

OBJECTIVES

Sea bass (Dicentrarchus labrax) has a growing significance in southern Europe and the Mediterranean basin. In 1997, the whole production was estimated at 23 000 t (1995: 14 000 t). Like any other species produced industrially, genetic improvement is a key aspect in the full development of the production sector. Until today, little attention had been given to this aspect.

Before initiating any selective breeding programme, it is necessary to evaluate the global genetic variability available. Concerning sea bass, this variability is mainly present in the wild, as no real domestication has occurred until now. The purpose of this project is, therefore, to identify the diversity existing among natural populations of sea bass in order to assess their respective performances and characteristics on profitable traits. It is what we call a testing programme.

RESULTS

The work realised in 1998 consisted in four meetings. During the first meeting, all partners were invited to decide jointly on the general organisation of the group. Four scientific meetings were planned during the two years of the project and a final meeting was scheduled to synthesise the conclusions and write the proposal. The discussed topics were listed and panels of experts were composed. Finally, the place and date of each meeting were fixed.

In the second session (‘Identification of commercial traits and strategies for a strain ranking’) a decision was made on which criteria the testing programme will be based. Here, producers were consulted to get the industrial point of view. In the report, the demands defining the fish market trends as well as the fish farmers’ requirements are presented. The consumers’ demand is heterogeneous between countries and the soaring role of supermarkets in the distribution chain is pointed out. A list of priority traits has been established taking into account both the consumers’ and the producers’ demands. It concerns the growth, quality and processing-related traits, disease resistance, survival, maturation, and malformations. The tools available to measure these traits are considered. Finally, the first steps of the testing programme are discussed. Expected genetic data are defined and required testing facilities and conditions are proposed. Different crossing designs are presented with their respective advantages and disadvantages.
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5.3.1. Genetics

The third meeting (‘Identification and evaluation of population variability’) called for the very last data concerning the structuring in space of wild sea bass populations. Different types of markers are already available on the species, and their characteristics are presented in the report. A differentiation (geographical and ecological) between populations has been revealed using allozyme and microsatellite markers. Based on microsatellites, genetic discreeteness suggests a differentiation between populations of the Atlantic Ocean and the Mediterranean Sea and the eastern and western Mediterranean basin. Based on allozymes, it is possible to discriminate between fish living in coastal and lagoonal environments. Care should be taken when choosing sites for sampling in relation to the biology of the species but also to transplanted populations. At least three places are recommended for sampling: in the Atlantic, and in the eastern and western Mediterranean.

During the last meeting (‘Strategies for comparison’), talks concentrated on the crossing design and the multi-site approach of the testing programme. The use of both industrial and laboratory structures was proposed. Discussions also focused on particular topics such as challenges to reveal possible resistance sources, the protection of the environment from ‘genetic pollution’ and the integration of new organisms such as clones and hybrids into the project. The minutes of this meeting are not included in the progress report, but will be presented in the final handbook.

The next meeting is scheduled for March 1999 in Italy. The topic to be discussed will be ‘Progenies rearing’. The amount of breeders to be kept and the zootechnical and genetic management of these stocks will be debated. The partners will standardise their rearing procedures from assisted fertilisation to the ongrowing stage. Further to these talks, a second session will gather together scientists and producers interested in the project in order to decide practically who will take part in the project and in what conditions. At this stage, the magnitude of the collaboration will determine the success of the multi-site testing programme.

The last meeting will take place in Spain in September. It will synthesise the conclusions of the other meetings. The proposal for realising a testing programme will be written.
FAIR-CT98-4314

Generation of a genetic body map for Atlantic salmon

OBJECTIVES

The main objective is to isolate and identify new genes from Atlantic salmon using a strategy based on cloning expressed sequence tags (ESTs). Messenger RNA (mRNA) will be prepared from nine adult salmon tissues (i.e. male and female gonad, liver, spleen, kidney, intestine, gill, brain, muscle) and used to generate cDNA libraries. Other tissues from salmon challenged with pathogens and salmon that are immunostimulated will also be included. Clones will be selected representing the abundant and rare mRNA transcripts in each library and 400–600 bp of their DNA sequence will be elucidated. Between 500 and 1 000 ESTs will be determined from each tissue, resulting in a detailed identification of the genes that are transcriptionally active in each chosen tissue. Selected ESTs (a minimum of 50 per partner), representing the most abundant mRNAs in each respective tissue and mRNAs of particular interest based on homology searches in the international databases, will be tested for their tissue-specific expression patterns among other salmon tissues.

RESEARCH TASKS

The Salgene partners will prepare poly(A) messenger RNA (mRNA) from the chosen salmon tissues and construct complementary DNA (cDNA) libraries using molecular genetic methods. Following this, the partners will determine partial DNA sequences of between 500 and 1 000 clones from each of the eight selected salmon tissues, which will be termed expressed sequence tags (ESTs). Homology searches will be performed between the salmon ESTs and DNA sequences deposited in the international DNA databases to identify as many salmon ESTs genes as possible. The Salgene partners will also perform tissue expression analysis on selected ESTs of interest to determine tissue expression profile data.
Recombinant vaccines against infectious pancreatic necrosis (IPN) in salmonid fish

OBJECTIVES

Our five-partner consortium (FAIR-CT-95-0353) is concerned with developing recombinant DNA vaccines for infectious pancreatic necrosis (IPN). IPN is now recognised as the most serious problem in salmonid farming in Norway and affects both juvenile and post-smolt fish. Recent estimates of losses due to IPN disease are approximately EUR 54 million per annum. A major part of our research initiative is the development of nucleic acid vaccines against IPN. To date, this has focused on the use of naked DNA constructs and packaged RNA viral particles harbouring the IPN VP2 gene. The VP2 gene product is known to be a protective antigen, and the world’s first recombinant subunit fish vaccine based on this protein purified from *Escherichia coli* has recently entered the market place. However, there is considerable evidence that conformational epitopes are important in protection and, thus, a bacterial product is unlikely to adopt the correctly folded structure. Nucleic acid vaccination circumvents this problem as the protein is made and folded in its natural host cellular environment. DNA vaccination is now emerging as an exciting technology in fish vaccine research. Several groups have shown that the cytomegalovirus (CMV) immediate early promoter works very efficiently when injected into fish muscle. As in other biological systems, injected DNA persists in the muscle tissue for a considerable time where it can stimulate extended antigen synthesis. It appears to exist as an episomal plasmid and does not integrate in the host genome. We have made recombinant plasmids harbouring the VP2 gene under the control of the CMV promoter and have tested their ability to express VP2 in cell culture. These plasmids have also been injected into fish muscle and expression and immunological analyses are ongoing. We are also investigating the ability of an alphavirus system based on a Semliki forest virus vector to deliver recombinant RNA. This system allows one to package RNA encoding VP2 and infect fish cells with suicide virus particles. Recombinant VP2 particles have been produced and are currently being tested in fish. The merits of DNA versus RNA delivery will be presented. In addition, the prospects for the use of genetic immunisation to combat fish disease and the likely public perception are discussed.

INTRODUCTION

Infectious disease poses the biggest single threat to aquaculture. The introduction of a new generation of both oil and non-oil adjuvants has greatly improved the efficacy of bacterial vaccines and has resulted in an impressive reduction in mortalities, especially against furunculosis. These adjuvants are
not without side effects, however, which can include visceral adhesions, and pathology and growth checks in vaccinated fish [7]. Current research is aimed at reducing this toxicity. Viral diseases remain an even greater problem for the industry. Recently, the world’s first recombinant subunit fish vaccine was launched in Norway against infectious pancreatic necrosis (IPN) [2] and a competitor whole virus vaccine has now also entered the market place. IPN is the only disease for which a commercial vaccine exists, although others are being developed. The most exciting development in vaccine research in recent years has been the emergence of the technique known as genetic immunisation (also known as naked DNA vaccination). This is based on the discovery in 1990 that naked plasmid DNA could be taken up by muscle (or, as subsequently shown, skin cells) and expressed in the host cell to produce the protein product encoded by that DNA (reviewed in [3]). This approach has been investigated in a wide variety of animal models and, where tested, has generally resulted in protection from challenge against the homologous disease agent. Typically, protective antigen(s) is(are) placed under the control of a strong eucaryotic promoter such as the cytomegalovirus (CMV) immediate early promoter that drives expression of the desired gene product(s). This technology has been tested in fish by a number of investigators with encouraging results. Recently, two groups have reported that vaccination of fish with CMV plasmids expressing protective antigens from infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicemia virus (VHSV) resulted in high-level protection against viral challenge [1, 4, 5]. Thus, there is little doubt that this technology represents the most promising approach to date in fish viral vaccine design.

We are investigating the ability of naked DNA plasmids under the control of the CMV promoter to deliver the IPNV protective antigen VP2 to fish muscle. cDNA encoding the VP2 gene has been isolated from a virulent Norwegian field isolate and cloned in a naked DNA expression vector. In parallel, we are also investigating the ability of a novel alphavirus delivery system, based on a suicide Semliki forest virus (SFV) vector, to deliver packed recombinant RNA molecules encoding VP2 to fish tissue.

**RESULTS**

DNA encoding the VP2 gene was generated using standard reverse transcriptase polymerase chain reaction (RT-PCR) techniques. This fragment was then cloned into the naked DNA vector pCDNA3 (Invitrogen) and the SFV expression vector pSFV1 ([6]; Figure 1). Clones were isolated with the VP2 gene in the desired orientation and expression was confirmed using a combination of indirect immunofluorescence (using a rabbit polyclonal antibody to the purified virus) of transfected baby hamster kidney (BHK) cell monolayers (data now shown) and immunoprecipitation of 35S-labelled polypeptides (Figure 2). The reactivity of the clones was also investigated using a panel of monoclonal antibodies to VP2, some of which were conformationally dependent [2]. The results of the latter experiments were somewhat variable, although, in general, the viral-encoded proteins generally reacted better than the plasmid-encoded DNA products. Expression was also confirmed in fish cell culture, although in the case of the SFV-encoded protein this was found to be temperature dependent, with little or no expression detected at growth temperatures below 20 °C.

**DISCUSSION**

The VP2 gene has been expressed in a naked DNA vector and a viral vector based on the Semliki forest virus replicon. Both constructs promoted the expression of a VP2 protein, which was recognised by polyclonal rabbit antiserum and in some cases by a panel of monoclonal antibodies to VP2. The SFV-encoded protein was the same size as the mature protein on the native virus. Expression has also been confirmed in fish cell culture for both constructs. However, expression from the SFV vector was temperature-dependent with little or no expression detected below 20 °C. This suggests that the SFV replicon does not function well in cell culture unless the growth temperature is sufficiently high. Experiments are under way to
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE
5.3.2. Health of aquacultured species

Investigate if SFV particles can function in fish tissue at the normal (lower) temperatures at which salmonids grow. The results of these experiments will determine the feasibility of the SFV approach in cold-water species.

We have recently initiated a vaccine trial in Atlantic salmon pre-smolts to test the efficacy of the naked DNA and SFV vaccine prototypes. The vaccines were administered by intramuscular and intraperitoneal injection, respectively. Samples...
have been taken for immunohistochemical analysis to detect expression of VP2. Serum antibody and T-cell proliferative responses are also being monitored. The ability of these vaccines to stimulate protective immunity following challenge will also be investigated.

It remains to be seen how the general public and licensing authorities will react to the concept of introducing recombinant DNA or RNA into fish tissue destined for human consumption. Naked DNA would appear to hold the most promise as a vaccine delivery system. Concern has been expressed that integration of foreign DNA into the host genome could lead to tumorigenic events in the vaccinated animal. However, this has not been detected in fish or in other vaccinated species to date. We believe that nucleic acid poses no threat to the consumer, even if it persists in fish tissue until the time of harvest. Thus, we see no reason why this technology cannot be applied in the construction of safe efficacious vaccines for the aquaculture industry.

ACKNOWLEDGEMENTS

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FAIR-CT95-0666

Molecular basis of fish immunity for disease resistance

OBJECTIVES

The objective of the project is to study the molecular mechanisms of trout defences against pathogens. The goals are:

- the generation of molecular markers predicting the capacity of fish immune response in relation to disease resistance;
- the generation of novel cell lines of the immune system for in vitro functional studies;
- the validation of these markers by gene regulation studies in vivo and in vitro;
- an interference with pathogen infection using genes encoding these markers, other genes involved in fish defences, and genes of the pathogens themselves.

The project also intends to create an industrial platform on fish genetics and diseases.

DESCRIPTION OF WORK

Task 1 consists of the homology cloning of cDNAs and genes encoding cytokine genes. Due to the relatively low conservation of cytokines, these genes will also be searched by differential cloning between stimulated and non-stimulated leucocytes.

Task 2 consists of the recognition and isolation of novel cell lines of the fish immune system, which are so far available essentially in the Channel catfish. To this end, four approaches are implemented, three of which rely upon gene transfer in vitro and in vivo.

Task 3 consists of the implementation of various functional studies of genes isolated in the project, to determine their role in the immune system and also to see whether a modification of their expression pattern can stimulate the fish defences. A major part of this task is to determine whether pathogen-derived protection can work in transgenic fish as it does in transgenic plants.

Task 4 consists of preparing for the creation of an industrial platform.

STATE OF PROGRESS

Homology cloning in the rainbow trout has resulted in full length cDNAs which encode the interleukin-1 beta, a chemokine receptor related to the receptor of interleukin-8, the cyclooxygenase 2 (COX2) and the proteasome
subunit LMP2 (upregulated in mammals by interferons). One genomic clone containing the interleukin-1 beta gene has also been obtained by PCR and characterised. Functional studies have already revealed potential roles for two of these factors in fish defences since: (i) three IL-1 beta transcripts are induced by infection with a bacterial pathogen and stimulation by PHA; (ii) COX2 is expressed in several lymphoid tissues and the cDNA contains motifs known to interfere with cytokine functions. Differential cloning from mitogen-stimulated and non-stimulated leucocytes has been attempted by several methods. Suppressive subtractive hybridisation looks more promising and has generated numerous candidate clones. Finally, the expression of the platyfish Xmrk receptor in a stimulatable form in mouse lymphocyte cell lines has revealed interactions with eight known genes of the cytokine-related intracellular signalling and two known cytokine-induced genes. One of these genes (JAK1) has been cloned in trout during this project.

Attempts to immortalise trout leucocytes by transfection with several known platyfish oncogenes have now been initiated, after earlier experiments have demonstrated that the platyfish Xmrk oncogene interacts with the signal transduction machinery of mouse lymphocytes. Peptides have been designed from the known sequences of the trout T-cell receptor and used to generate monoclonal antibodies potentially able to mark trout T-cells. So far, none of them recognises a substantial fraction of peripheral blood cells or thymocytes. As an alternative and original approach, experiments have shown that regulatory elements of a mammalian T-cell specific gene (CD2) drive the expression of a reporter gene in the thymocytes of transgenic troutts. Stable lines of such fish are now generated, and transgenic troutts expressing an exogenous transmembrane protein on their T-cells are tentatively produced. Candidate regulatory sequences from the trout TCR genes are also cloned in parallel. Finally, an important methodological investment has been made to increase the feasibility of enhancer trap screens to identify new cell populations of the medaka immune system and has resulted in: (i) new possibilities to detect transgenics even at low frequencies by transfer of the tyrosinase gene; (ii) considerable improvement of the cell reimplantation techniques for future *in vitro* enhancer traps; and (iii) two minimal promoters which will be incorporated into enhancer trap constructs.

Functional studies of several genes cloned in the project already permit novel cytokine-based strategies to positively manipulate the trout immune system by either genetic or non-genetic routes to be foreseen. Several ‘natural’ or mutant versions of sequences encoding variable regions of MAb reacting with the VHSV G protein have been cloned and used to engineer single-chain antibodies (scAbs). Some of these molecules were shown to bind and even neutralise several VHSV strains, and are currently used for passive immunisation of normal fish and for transgenic experiments. Transfection of fish cell lines with vectors, including two VHSV genes in sense orientation, has resulted in expression of the encoded proteins. These vectors have been successfully used for DNA vaccination and are currently being tested in transgenic experiments.

The four industrial subcontractors have prepared the basis of a platform in the field of fish genetics and diseases, and a workshop associating scientists and the fish farming industry.

**MAJOR ACHIEVEMENTS**

- Cloning/identification of several trout cytokine, cytokine-related and cytokine-induced genes.
- Stable transgenic troutts expressing a reporter gene in thymocytes; methodological bases for an enhancer trap screen in medaka.
- Evidence for the implication in the immune response of several genes cloned in the project.
- Engineering of several reactive single-chain antibodies against the VHSV G protein.
- Expression of several VHSV gene constructs in fish cells; similar constructs already efficient for DNA vaccination; transgenic troutts available with an antisense construct derived from the VHSV M1 gene.
- Basis for an industrial platform in the field of fish genetics and diseases.
**5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE**

5.3.2. Health of aquacultured species

**FAIR-CT95-0850**

*Salmonid protection in aquaculture: development of a fish herpesvirus as a vector for vaccination*

**OBJECTIVES**

The final goal of this proposal is to develop a vaccination vector which is useful in aquaculture, leading to protection of salmonids against viral diseases such as those associated to rhabdoviruses (VHS — viral haemorrhagic septicaemia and IHN — infectious haematopoietic necrosis). The fish model is the rainbow trout *Oncorhynchus mykiss*. The initial proposal was a four-year programme, including four main tasks: (1) to localise potential insertion sites in the herpesvirus salmonids (SaHV-1) viral genome (use of reporter gene); (2) to study the virulence of the corresponding SaHV-1 recombinants; (3) to construct recombinants expressing heterologous viral antigens; and (4) to perform *in vivo* assays of the recombinants. According to comments from the Commission, the work programme was reduced to a period of two years, and the objectives were refocused as follows:

1. to generate a viral replicating vector based on the herpesvirus salmonids;
2. to study the safety on this recombinant by evaluating its loss of virulence (*in vivo* assays in rainbow trout).

**WORK AND ACHIEVEMENTS**

*Preliminary steps*

The achievement of the first objective of our programme required several preliminary steps that were all initiated during the first year of the programme:

1. sequencing of the SaHV-1 genome in order to identify potential insertion sites for foreign genes;
2. construction of an intermediate plasmid that will be used to generate SaHV-1 viral recombinants;
3. production and purification of viral DNA required for generating SaHV-1 recombinants;
4. production of viral stocks required for fish immunisations;
5. determination of the optimal experimental conditions for transfection experiments with the available plasmid.

The essential components of these different tasks foreseen for the year of the contract were accomplished, with a few modifications that were discussed during our coordination meetings. These meetings were organised every six months. Each meeting was followed by a short report written by the coordinator and sent to each of the other partners. The results obtained during the first reporting period can be summarised as follows:
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

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1. Sequencing of the SaHV-1 genome allowed the identification of a potential insertion site within the SaHV-1 genome. This target gene is the counterpart of CCV gene 49 encoding, deoxynucleoside triphosphatase (dUTPase). It constitutes an attractive insertion site for generating a viral recombinant since it is known to be non-essential in mammalian herpesviruses and to have a role in pathogenicity.

2. An intermediate plasmid was constructed by inserting a reporter gene within the dUTPase gene of the SaHV-1 genome downstream of the CMV immediate early promoter. The reporter gene that we chose is the boalactosidase coding gene.

3. SaHV-1 DNA required for generating viral recombinants was purified from a virus grown in monolayer rainbow trout cells. The yield of DNA production was initially relatively low, but recent yields were significantly higher probably as a result of continued passage of the RTG-2 cell line and perhaps also due to a greater incubator temperature stability.

4. Viral stocks were produced for in vivo experiments, as a wild-type virus is required as a positive control in virulence studies and for immunisations of adult rainbow trout in order to obtain specific antisera. These immunisations were not foreseen in the initial work plan but were decided at our first coordination meeting.

5. Transfections of RTG-2 with the available intermediate plasmid were initiated. Transfection efficiency was too low to envisage efficient co-transfections with both plasmid and viral DNA. It was decided to test other fish cell lines as EPC (Epithelioma papulosum cyprini) and CHSE-214 (chinook salmon embryo) cells. EPC cells are not susceptible to SaHV-1 infection but might be used in a first step of co-transfection with plasmid and viral DNAs followed by a second step of infection of susceptible cells. However, CHSE-214 cells seemed to be the best candidates for obtaining SaHV-1 recombinants.

In vivo experiments

The achievement of the second objective of the programme also required a preliminary step of determining optimal experimental conditions for in vivo assays in rainbow trout. Virulence studies were initiated in February 1997. The purpose was to evaluate the virulence of a wild-type virus administered by bath delivery. Virulence appeared as moderate but real on young fish (27 % of mortality after 50 days), showing that infection by SaHV-1 is effective by bath delivery for juvenile rainbow trout. For older fish, neither symptoms nor mortality was observed although mortality is effective when SaHV-1 is administered by injection instead of bath delivery (data not shown).

In terms of in vivo assays, immunisations of adult rainbow trout were also performed for producing antisera that were tested in neutralisation assays and immunofluorescence. As few of these sera showed neutralising activity and that titres of neutralising antibodies were very low, several approaches were discussed to improve the immunisation efficiency, and it was decided to perform rabbit and mice immunisation using a recombinant purified SaHV-1 protein. It was proposed to use as an immunisation antigen a recombinant protein expressed in E. coli and containing a part of the gp46 glycoprotein. A 0.7 Kb fragment containing a part of the CCV homologous-ORF46 sequence was isolated from a cosmid clone and inserted into an expression vector in order to produce a recombinant protein fused at its NH2-terminus to six histidine residues. Expressed protein was purified and inoculated in mice and rabbits to produce a specific antiserum.

Second period of the programme

According to the results obtained in the preliminary steps described above, the following tasks were continued during the second period of the contract:

1. Sequencing the SaHV-1 genome was continued in order to identify another potential insertion site for foreign DNA, particularly the TK locus that was not yet identified at the end of the first year. Analysis of the genomic fragment previously identified indicated the presence of genes corresponding, in order, to open reading frames (ORFs) 57 (DNA
5.3. BIOL OGY OF SPECIES FOR OPTIMISATION
OF AQUACULTURE

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polymerase), 58, 49 (dUTPase) and 48 of the Channel catfish virus. This confirmed that the genomes of SaHV-1 and CCV are collinear.

Sequences of 857 M13 templates were read and entered into the database. These cover about two thirds of the SaHV-1 genome. Sequences were analysed in order to identify the TK locus, their conceptual translation products being screened for similarity to TKs of other herpesviruses. The TK gene was still not detected at the end of this research programme.

2. SaHV-1 DNA required for generating viral recombinants was purified regularly from batches of purified virions and for an extracellular virus. DNA preparations were used in transfection experiments.

3. Stocks of the wild-type SaHV-1 virus to be used in virulence studies were also produced regularly. A part of this virus was also used for infection of cells further transfected with the plasmid containing the sequence of interest.

4. Virological and histological studies were performed on fish harvested from in vivo experiments. Virological analyses were done on the RTG-2 cell line but, surprisingly, any cytopathic effect was observed in young as well as in older fish. A regards histopathology, some moderate lesions were observed in fish samples harvested late in the infection, particularly in young fish. Lesions appeared the most often in the liver, sometimes in the gills, and once in the kidney. In older fish, lesions were only observed in the gills.

5. Many attempts of transfecting RTG-2 cells (rainbow trout gonad cells) were made using several methods and numerous conditions, but without success. It was thus decided to try another cell line for transfections: CHSE-214 (chinook salmon embryo) cells. CHSE-214 fish cells could be transfected with the plasmid DNA; however, they were totally refractory to the entrance of SaHV-1 DNA. Any recombinant virus could be isolated. In fact, any cytopathic effect was observed after transfection with viral DNA, even when SaHV-1 DNA was used alone in simple transfection experiments instead of co-transfections. An alternative method was assayed that consisted of transfecting with the plasmid SaHV-1 infected cells, at successive post-infection times (24, 48 and 72 hours). In this case, infection was effective; however, any transfected cell could be revealed further.

CONCLUSIONS

Characterisation of the SaHV-1 genome structure has allowed the choice of an attractive potential insertion site for foreign DNA, which is the dUTPase coding gene. The choice of this site as a primary target for insertion of foreign genes opens the possibility of producing additional mutations in the TK gene (when it is identified), thus providing a means of further attenuating the virus. The genome of SaHV-1 is similar in structure to those of several mammalian alphaherpesviruses, such as the pseudorabies virus (of pigs), bovine herpesvirus 1 (of cattle), equine herpesvirus 1 (of horses) and the varicella-zoster virus (of man), and different from that of the Channel catfish virus. However, SaHV-1 is much more closely related genetically to CCV than it is to mammalian herpesviruses. This supports the view that similar genome structures have arisen independently during herpesvirus evolution. Evidence that the genes in SaHV-1 and CCV are ordered differently mirrors the situation in the three subfamilies of mammalian herpesviruses, where conserved genes are present in several rearranged blocks, and thus provides important data for classification of herpesviruses of lower vertebrates.

Work aimed at determining the complete DNA sequence of SaHV-1 was continued during the second year of the contract. A preliminary analysis of the SaHV-1 genomic sequence, including the sequence of the dUTPase gene, was completed and accepted for publication (Journal of Virology, March 1998). Efforts to locate the TK gene by translational screening were made while the sequencing was in progress. To date, this gene has not yet been identified. There are three possible reasons why the TK gene has not been detected. Firstly, the genome may not encode a TK. This appears unlikely, since the virus is sensitive to nucleoside analogues that are activated by known herpesvirus thymidine kinases. Secondly, none of the random sequences analysed to date may have originated from a conserved region of TK (about one
third of the genome is not currently represented in the database). Thirdly, TK may be poorly conserved in SaHV-1, so that even if some of the random sequences did originate from the TK gene, the gene would not be recognised. TK is known as a rather poorly conserved protein; only a few residues are identical in all known herpesvirus TKs.

The final goal of this programme is to use SaHV-1 as a vector for vaccinating salmonids against viral diseases such as those associated with rhabdoviruses. The use of a reporter gene as the one encoding β-galactosidase is an intermediate step that should facilitate selection of SaHV-1 recombinants. We constructed the pYub-gal plasmid, which was specifically designed to allow easy insertion of genes of interest in place of the reporter gene.

Transfections were initiated as soon as this plasmid was available. Great difficulties were encountered due to the very low transfection efficiency of RTG-2 cells. Other cell lines were tested in transient transfection experiments, and promising results were obtained with CHSE-214 cells. Optimal experimental conditions were determined to transfect CHSE-214 with this plasmid. However, cells were completely refractory to the entrance of SaHV-1 viral DNA. Attempts to avoid this problem were made by using an alternative procedure consisting of transfecting cells previously infected with SaHV-1. All attempts were unsuccessful. This lack of success in a crucial step of the programme obviously led to a ‘bottleneck’ in our initial plan. The initial purpose of testing the virulence of at least one recombinant in fish was thus seriously compromised.

In terms of in vivo experiments, most of the work focused on the study of virulence of the wild-type SaHV-1 for rainbow trout. Adult rainbow trout immunisations were also carried out in order to produce antisera. These antisera were tested in indirect immunofluorescence assays. After testing, any of the rainbow trout antisera obtained could be considered as a good immunological tool. Alternatively, immunisations of rabbits and mice were conducted using a recombinant protein as immunisation antigen. A part of the SaHV-1 gene corresponding to CCV ORF46 was cloned and expressed in E. coli, and the purified protein was inoculated into mice and rabbits in order to produce specific antisera.

Virulence studies were conducted with the wild-type SaHV-1 and the possibility of using bath delivery as compared with individual fish injection as the administration route was evaluated. SaHV-1 virulence appeared as moderate but real in young fish (27% of mortality after 50 days), showing that infection by SaHV-1 is effective by bath delivery for juvenile rainbow trout. The symptoms observed after infection were extremely crude; the fish died in less than 24 hours. Neither symptoms nor mortality appeared in older fish. To our knowledge, this was the first demonstration of the SaHV-1 virulence for juvenile rainbow trout using this route of administration.

Downstream of these in vivo experiments, virological and histopathological analyses were conducted. Some of these studies should be confirmed and completed, in particular the virological analysis which was highly surprising since no virus could be detected in the cell culture despite the fish mortality observed after SaHV-1 administration by bath delivery. Histopathological studies indicated the appearance of some lesions in fish samples harvested late in the infection, particularly in young fish. None of the fish analysed within two weeks’ post-infection shows a specific histopathological lesion. Lesions appeared most often in the liver with a moderate hepatocyte necrosis, sometimes in the gills with a widespread epithelial necrosis and pyknosis of the secondary lamellae, and once in the kidney with lesions showing scattered necrosis and pyknosis of epithelial cells of the renal tubules.

Pathogenicity of SaHV-1 was only described after inoculation by individual injection. Mortality rates observed in our challenge experiments demonstrate the viability of bath delivery as the administration route of herpesvirus salmonids (SaHV-1) to rainbow trout. This obviously constitutes a crucial prerequisite for using SaHV-1 as a potential vector for vaccinating salmonids.
FAIR-CT96-1615

Biology and management in the control of lice on fish farms

OBJECTIVES

Summary

This concerted action aims to increase communication between scientists and industry so as to achieve more effective long-term control of sea lice on salmon farms. It includes: (i) the establishment of a register of persons interested in sea lice biology and control; (ii) a computerised network using the Internet (e-mail and world wide web); (iii) circulation of a newsletter twice a year; and (iv) production of a computerised bibliography of relevant publications. Over three years, a series of European workshops or conferences will be held on farm management to control lice, lice biology, host defence mechanisms, cleaner fish technology, and integrated lice control systems. Workshop proceedings will be published. Participation in activities is open to industry and all scientists actively conducting research which will contribute to the control of lice on fish farms.

Introduction

Lice are the most serious parasite on marine salmon farms in Europe. The lice control methods which are being used and/or developed include chemotherapeutics, cleaner fish, vaccines and farm management, and it is increasingly clear that a suite of alternative lice control strategies are necessary. The development of these methods benefits from improved understanding of the biology of lice, cleaner fish and natural host defence mechanisms. The need to control lice is urgent and the pace of research is increased by communication, including the bringing together of researchers at meetings. Communication between researchers from a wide range of disciplines will aid the finding of an environmentally acceptable way of controlling lice, and this may have benefits in treating other parasites and in the further understanding of parasites and fish biology. The broad aim of this concerted action is to increase communication between scientists and industry so as to better manage and avoid lice infestations on fish farms.

METHODS

Communication between researchers is being promoted through the establishment of:

- a register of persons interested in the biology and control of sea lice;
- an information page on the world wide web linking to the newsletter and bibliography;
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

• an open e-mail discussion group on the Internet;
• a newsletter to be circulated twice a year;
• a computerised bibliography of relevant publications; and
• a series of European workshops or conferences.

Responsibilities for the workshops and conferences are divided between the main partners, while the coordinator leads the other tasks.

Workshops compare methodologies, discuss the results of recent research and identify the needs of future research. From each workshop, selected papers will be published in an internationally available publication (e.g. in the newsletter, industry magazine, scientific periodical or as a book). At the end of the project, a comprehensive review will be produced by the coordinator. This will include a review of sea lice control options, their strengths and weaknesses, and key areas for further research.

RESULTS

Register of persons interested in sea lice biology and control

At the start of the project a register of ‘researchers’ was compiled by the project partners from previous workshops, conferences and contacts in research and industry. This preliminary document listed over 170 persons with interests in sea lice control. Each person was sent a letter along with a copy of the first newsletter on 19 May 1997 explaining the nature and objectives of the project and asking them to confirm or make corrections/additions to their interests and contact details. In developing the register it became apparent that many persons were more involved in management of lice on farms or involved in aquaculture regulation and were not directly involved in research (Table 1). Thus the register is now more accurately called a list of persons interested in sea lice control. On 3 October 1997 a follow-up letter was sent either by post or e-mail to the persons who had not confirmed their contact details to remind them to return the information. All those who did not reply were eliminated from the list. We believe that those who did not reply were either no longer at the address given or were no longer actively interested in the subject.

At present there are 169 individuals from 19 countries (Table 1) on the register and requests to join continue to be received. This data is stored on a Microsoft access database and a hard copy of the requests/contact details is also kept on file. The database was designed to store names, addresses and other details. The database also facilitates the easy production of the register in hypertext mark-up language (HTML) format for the world wide website. It enables pages to be quickly and routinely updated directly from the database, and allows visitors to link directly to subscriber homepages or send them e-mails directly. Address lists and mailing labels can also be produced directly from the database. New subscribers can join the register by filling out an electronic form at the project WWW site or by sending the following information to the coordinator (EcoServe): name; address; telephone number; fax number; e-mail address; position; interests; homepage (WWW).

Newsletters

Three newsletters have been produced to date. The first newsletter was produced in April 1997 and was sent to all persons on the preliminary list. It explained the background to the project and gave information on the meeting to be held in Norway in November. After the meeting in Norway, two further newsletters were produced. The first of these (Caligus 2) included a report on the Trondheim workshop on sea lice control on fish farms includ-
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ing the list of delegates, news of the new Internet discussion group on sea lice biology and control, the current mailing list of persons interested in sea lice biology and control, information on the 1998 international symposium on sea lice biology, a notice of an initiative in Canada to develop a lice control strategy, and some papers and abstracts from the workshop. The latter included papers entitled ‘Best current practice for lice control in Norway’, ‘The effect of swimming depth, speed and artificial light treatment in Atlantic salmon (*Salmo salar*) culture on the infestation of the copepod *Lepeophtheirus salmonis*’, and ‘Lice fighting the environmentally friendly way’.

The third newsletter was devoted to the bibliography of publications relevant to sea lice control on fish farms. The newsletters are available on the world wide website and by post from EcoServe.

**Bibliography**

The bibliography was compiled from EcoServe’s existing aquaculture bibliography, literature searches, and information supplied by researchers. All the citations of relevant papers are stored in one file in an EndNote® bibliographic database. This stores all the information in standard format and facilitates the easy production of the bibliography in hypertext mark-up language format for the world wide website. In the bibliography in the third newsletter, 464 relevant publications were listed.

**World wide website**

A world wide website has been established (http://www.ecoserve.ie/sealice) to host the concerted action homepage. The information available on the site includes the HTML format of the project newsletter *Caligus*, details of workshops and conferences, the register of persons interested in sea lice biology and control, a bibliography of sea lice literature as well as contact details and an outline of the project. A counter is included to keep track of the number of people visiting the site. The site is routinely updated to incorporate new additions to the register of researchers and bibliography.

**E-mailing discussion list**

An electronic listserver group has also been established to facilitate discussion between industry, researchers and subscribers. This is open to all those with Internet access. To join the list, the subscriber must send an e-mail to Listserv@listerv.hea.ie, leaving the subject line blank, with the message ‘subscribe Caligus firstname surname’ in the main body of the message. To send an e-mail to all those who subscribe to the list, you send your message to Caligus@listserv.hea.ie and the mail is distributed to the people who have subscribed to the list. Various other commands will allow you to check the list to see who else is signed up. Currently there are 135 subscribers.

**Workshops and conferences**

About 50 delegates attended the workshop in Trondheim from 6 to 9 November 1997. The audience was a mix of researchers, government scientists, fish farmers and fish food manufacturers from Norway, Scotland, England, Ireland, Canada and Japan. This led to discussions ranging from the longer-term visions of solutions to sea lice problems on fish farms, to the immediate needs of farmers in dealing with infestations. Arising from the papers presented and discussions between participants at the workshop, articles have been published in *Caligus* 2 and 3. Further documents are in preparation and will be published in *Caligus* 4.

Two workshop presentations provided a review of current biological knowledge on lice, lice–host interactions, and host defences, with a view to developing improved control methods in the long term. The first keynote presentation by Dr
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5.3.2. Health of aquacultured species

Geoff Boxshall, one of the foremost experts in copepod biology in the world, reviewed recent discoveries in copepod biology which have relevance to lice biology. Better knowledge about how lice find and choose a mate may enable the development of methods to disrupt this behaviour (e.g. trap lice, new treatments). Current studies on the interactions between lice and their host at a chemical level were described by Dr Stewart Johnson. Getting to the exact nature of such interactions will help improve a fish’s defences against lice.

The more immediate issues of dealing with sea lice were introduced by speakers from industry. Subsequent keynote presentations by Pelle Kvenseth (HydroSeafood and KPMG) and Jim Treasurer (Marine Harvest McConnell) summarised the development of the lice problem in Norway and Scotland, and how it was being handled in each country at present. David Jackson (Marine Institute, Ireland) provided a paper describing the lice management measures in place in Ireland. Following these presentations, the audience broke into smaller discussion groups which discussed the merits of different lice treatment methods, and the best overall lice control strategy today. The results of these discussions will be published in Caligus 4.

Methods for sampling lice as part of monitoring levels on farms were outlined by Pelle Kvenseth and David Jackson, with additional input from Per Andersen, Jim Treasurer, Karin Boxaspen, and Mark Costello. These methods were discussed in some detail, demonstrated on an excursion to a fish farm, and will be published in Caligus 4.

DISCUSSION

The main achievements of the project to date have been:

- the establishment of a register of 169 persons interested in the biology and control of sea lice;
- the production of three newsletters;
- the compilation of a bibliography of sea lice literature;
- the establishment of a world wide website which includes a project description, researchers’ contact details, bibliography, and details of meetings;
- the establishment of an electronic listserver discussion group on sea lice biology and control;
- the holding of an industry-researchers’ workshop in Trondheim, Norway, from 6 to 8 November 1997.

Over the next two years, the project will:

- continue updating the register of persons’ details;
- expand the bibliographic database to include keywords and summaries of publications;
- produce further newsletters (Caligus 4 is in preparation and will include further abstracts and papers from the Trondheim conference);
- update the website and add links to related websites;
- manage the listserver e-mail discussion group;
- hold a scientific conference in Amsterdam, the Netherlands, in July 1998;
- publish the proceedings of the Amsterdam conference;
- hold a combined conference and workshop in Dublin, Ireland, in 1999 and publish its proceedings.

The three-day project conference in Amsterdam, from 22 to 24 July 1998, will comprise oral and poster presentations concerning recent advances in the biology and control of sea lice. The plenary session speaker will be Mr Gordon Rae.
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of the Scottish Salmon Growers’ Association. This will follow the very successful formula of the first sea lice conference held in Paris in 1992, and takes place during the Fourth World and Third European Crustacean Congresses hosted by the University of Amsterdam from 20 to 24 July 1998. This will enable delegates attending the sea lice sessions to experience crustacean research within its wider context, to interact with the top crustacean specialists worldwide, and to hear about research in important related topics, such as shellfish aquaculture and fisheries. The proceedings will be peer reviewed and published after the meeting. This sea lice meeting is being organised by Dr Geoff Boxshall on behalf of the partners in this EU concerted action.

The theme of the third and final project meeting to be held in Ireland will be ‘Integrated lice control’. This will include topics such as host defences, lice biology, management practices, use of chemotherapeutants, fallowing, and wrasse, and will involve speakers from industry, aquaculture management and research. The project welcomes and invites suggestions as to the scope, format and content of this meeting that will be finalised following the Amsterdam conference. The project can sponsor the attendance of European researchers without funding at meetings.

Table 1: The occupation and country of origin of the persons on the project contact register (in addition to the countries in the table, there are also single subscribers from the Republic of China, the Republic of Korea, Russia, Sweden, the Netherlands, Australia, Denmark, and the Faeroe Islands)

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<th>Occupation</th>
<th>Occupation (%)</th>
<th>Country of origin</th>
<th>Number of subscribers</th>
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<td>Fish veterinarian</td>
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</tr>
<tr>
<td>Aquaculture consultant/adviser</td>
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<td>Japan</td>
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</table>
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

FAIR-CT96-1840

Investigation and quantification of the stress associated with accumulation of carbon dioxide in eel farms with recirculating water

OBJECTIVES

The aim of this project is to identify and quantify the physiological and oxidative stress associated with exposure to chronic hypercapnia in eels grown under intensive conditions in closed-cycle fish farms, and the effects this stress has on performance in culture. Hypercapnia occurs because carbon dioxide produced as a metabolic end product accumulates in the recirculating water. It is well established that hypercapnia is a physiological stressor because it causes impaired blood oxygen transport and tissue acid-base imbalances, and there is evidence that it could lead to oxidative stress in eel flesh, with the consequent destruction of nutritionally valuable polyunsaturated fatty acids and production of free radicals. Stress compromises growth and hence the economic viability of the fish farm, and oxidative stress influences the nutritional value of the product. The project will provide guidelines for the degree of hypercapnia that does not influence the performance and nutritional value of eels grown under intensive closed-cycle conditions.

DESCRIPTION OF WORK

This project uses an automated computerised system to expose eels to a series of accurately controlled levels of chronic aquatic hypercapnia (task 1). Experiments will then be performed to identify and quantify the physiological stress associated with this chronic exposure (task 2), and to evaluate the oxidative stress in eel flesh through measures of lipid composition and various antioxidant parameters (task 3). These analyses of physiological and oxidative stress will be correlated with specific indicators of performance such as growth and feed-conversion efficiency (task 4). These investigations will not only be performed on eels exposed to a constant degree of hypercapnia but also to diurnal cycles of fluctuating hypercapnia such as may result from feeding regimens. Close collaboration amongst participants and effective integration of research activities are assured by the fact that almost all of the experimental work will be conducted at one site, the La Casella Fluvial Hydrobiology Research Station (formerly the Experimental Thermal Aquaculture Plant) run by ENEL-Ricerca in northern Italy.

STATE OF PROGRESS

A significant amount of progress has been made during the second 12 months of the project. As described in the first annual report to the Com-
mission, a decision was made to perform growth studies on small animals for shorter periods and then separately to adapt large animals to hypercapnia for physiological studies (the original work plan foresaw maintaining small animals within the growth study until they reached a size suitable for physiological experimentation). Within the new approach, during the second 12 months, populations of small eels have been chronically exposed both to fixed levels (PWCO2S: 0, 15 ± 1, 30 ± 1 and 45 ± 1 mmHg) of hypercapnia and to fluctuating levels (PWCO2S: 0, 15 ± 10, 25 ± 10 and 35 ± 10 mmHg) of hypercapnia (task 1), and their growth and mortality rates measured (task 4). For reasons linked to internal restructuring at the La Casella facility where the eels are maintained, the growth study on small eels exposed to fluctuating levels of hypercapnia was initiated and completed immediately after the growth study on small eels exposed to fixed levels. Investigation of oxidative stress (task 3) and feed acceptance (task 4) was completed on the small eels chronically exposed to the fixed levels of hypercapnia. Tissues were collected from the small eels chronically exposed to fluctuating levels of hypercapnia and are presently being analysed to describe the presence of oxidative stress (task 3).

Following completion of the growth studies, large eels were chronically exposed to fixed levels of hypercapnia (task 1) and the physiological effects of this described (task 2; the analysis of results is only partial at present). Furthermore, an additional series of experiments on the physiological effects of acute hypercapnia exposure were performed (within task 2).

ACHIEVEMENTS

Although the sequence of our tasks has been modified (for the reasons stated above) and hence some of the deliverables have not been achieved according to the timetable described in the technical annex, very significant progress has been made. The studies on the effects of chronic exposure to fixed hypercapnia have been almost entirely completed, with the exception of one sub-task (4c) and some data analysis within the sub-tasks of task 2. These latter measurements and analyses will be completed by spring 1999. The results to date indicate that eels can compensate for the physiological effects of mild hypercapnia (PWCO2 = 15 ± 1 mmHg) without any negative effects on growth. More severe hypercapnia (PwCO2S = 30 ± 1 or 45 ± 1 mmHg) is tolerated but causes significant physiological stress and inhibition of growth. There appears to be little oxidative stress associated with chronic fixed levels of hypercapnia.

The effects of exposure to fluctuating hypercapnia on growth have also been investigated, and revealed similar trends to those seen with fixed hypercapnia.

FUTURE ACTIONS

In the first part of the third year of the project, the participants will focus on completing the data collection and analyses within tasks 2 and 4 for eels exposed to fixed hypercapnia. The remaining part of the year, up to month 36, will focus on completion of task 2 (‘Physiological effects of hypercapnia’), task 3 (‘Oxidative stress associated with hypercapnia’) and task 4 (‘Effects of hypercapnia on performance’) on large eels chronically exposed to four fluctuating CO2 levels. These will be completed and the results reported at month 36.
FAIR-CT97-3406

Improved diagnosis of Gyrodactylus parasites infecting aquacultured species

OBJECTIVES

To improve the diagnosis of Gyrodactylus parasites infecting aquacultured salmonid species in Europe, to provide information on the degree of variability in and reliability of genetic and morphological factors used to identify Gyrodactylus species, and to compare pathogenicity of Gyrodactylus salaris from different areas of Europe.

This project aims to produce criteria for Gyrodactylus diagnosis, integrating pathogenicity, morphological and molecular investigations. Such integrated studies have been recommended by the leading expert on Gyrodactylus (Malmberg and Malmberg, 1993) and are viewed as the best approach for modern parasitology. This project will carry out the first such work and will provide a sound basis for the design of sampling and identification programmes for this important fish pathogen.

Gyrodactylus salaris is a parasite that can infect several species of salmonid fish and feeds on host mucus and skin. In general, Gyrodactylus infections of wild fish are of limited pathogenicity but the effect of G. salaris on populations of wild Atlantic salmon in Norway has been a notable exception. G. salaris has resulted in the almost total depletion of juvenile salmon in affected Norwegian rivers. The original source of G. salaris in Norway is believed to be fish from an infected hatchery and, apart from the Baltic region, all reports of G. salaris in Europe are from farmed rainbow trout. The parasite spread rapidly within Norway as fish were moved to stock farms and rivers. To date, 39 rivers and 37 hatcheries have been infected. Eradication of this parasite from aquaculture facilities is normally achieved by destocking and disinfection of the facility. In rivers, an expected increase in the survival rate among salmon pairs over time does not seem to occur, possibly because native fish with no previous exposure to G. salaris are frequently introduced to the pathogen through reproduction of strayers from other rivers and escapees from fish farms. In some of the most badly affected rivers, the salmon population has become extinct. So far, 23 rivers have been cleared by rotenone treatment to kill all fish and restocked with parasite-free fish. Rivers are intensively studied for five years before they are declared free from G. salaris. At present, 10 rivers have achieved this status while 11 are in the process. In two rivers the parasite has reappeared after the treatment. G. salaris has resulted in losses of over 25% of the total wild salmon catch in Norway and the eradication programme currently costs NOK 3.5 million per year. Such drastic eradication
techniques in river systems may not be economically or ecologically viable in other areas of the Community and closure and drying to clear farms of infection would have severe economic impact.

*G. salaris* has been demonstrated to be at least equally pathogenic, if not more so, to salmon from European origins other than Norway. Large and economically important salmon populations exist in the UK, Finland and Russia and are at risk from the importation of *G. salaris* with live fish, particularly rainbow trout. This strongly indicates the necessity for strict controls to prevent further spread of this parasite in Europe.

Commission Decision 96/490/EC allows for one year a ban on the import of live salmonids from fresh water into the UK and Ireland unless they are from areas free of *G. salaris*. It is also an expressed intention of the Commission to introduce a proposal to change the status of *G. salaris* from a list I to a list II category disease under Community Directive 91/67/EEC. This is in recognition of its status as a disease that is present in some parts of the EU but exotic to others and because of its severe economic implications.

In order to implement the surveillance testing and laboratory examination required in order to achieve approved zone status under Directive 91/67/EEC, reliable and practical methods of pathogen diagnosis are required and guidelines need to be given on the criteria used for pathogen identification.

To date, there are no such guidelines on the identification and discrimination of *Gyrodactylus* within Europe and, in particular, on the identification of pathogenic forms. Examination of the shape and size of the opisthaptoral hooks has been the peer-accepted method used in *Gyrodactylus* species descriptions and identifications. However, species differences in morphology can be very slight and structural similarities in different species can complicate identification. In the case of *G. salaris*, identification can be difficult as the morphological features used show wide variations within a species and may overlap with other species. Such variations have prompted the search for improved methods of identification such as SEM study of the hooks. However, these methods may not be suitable for routine use in a diagnostic laboratory. Implementation of routine monitoring for pathogens requires reliable and straightforward methods which can be readily applied throughout the Community. The development and improvement of diagnostic methods is a dynamic process involving application of new techniques and detailed knowledge of the reliability of features used. In order to assess the probability of misidentification of gyrodactylids, it is important to know the degree to which diagnostic characteristics overlap between species.

New molecular genetic techniques have been applied to the study of *Gyrodactylus*. Examination of the ribosomal RNA gene array (rDNA) has led to the development of DNA probes and restriction fragment length polymorphisms (RFLPs) to discriminate *Gyrodactylus* species commonly found on salmon and trout in Europe. However, the regions of rDNA studied so far are not sufficiently variable to reveal differences between *G. salaris* and *G. thymalli*, two species that have different host specificities and pathogenicity but are difficult to separate by morphology alone. The ribosomal spacer region has been found to be more variable between closely related species than the ribosomal genes themselves. Examination of more variable regions such as the external transcribed spacer and non-transcribed spacer regions of rDNA are required to locate nucleotide differences between *G. salaris* and *G. thymalli*. The technique of random amplified polymorphic DNA (RAPD) has also been applied to the study of gyrodactylids. This provides an alternative method of locating polymorphic DNA of potential value in diagnostic tests and can be used to examine the degree of similarity or relatedness between species or strains of parasite.

To date, no studies have been carried out to investigate the variation in genetic factors within species of *Gyrodactylus* infecting aquacultured fish species or fish inhabiting watercourses which may provide a source of infection for aquaculture facilities. Knowledge of such variation and of any diagnostic molecular features common to more than one species is vital in order to assess the probability of misidentification, to validate new methods of identification and to avoid false results.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

FAIR-CT97-3413

Development and use of cell cultures for commercially important aquatic invertebrates

OBJECTIVES

Farmed crustaceans and molluscs account for approximately 60% of the world's consumption of these groups. The organisms represent high value (in economic terms), and are very important to the economies of France, Portugal and the Far East. Production is likely to increase substantially over the next 10 years. Yet, production is blighted by disease and a lack of understanding of physiology, leading to high economic losses. This project addresses some of the fundamental issues regarding the biology and pathology of species of commercially important invertebrates. In particular, the development of cell lines will have an immediate impact on the ability to study viral diseases, which are often suspected to occur but which cannot be confirmed in laboratory studies. Also, well-characterised cell lines will enable the study of exotoxicology, pathology, and disease control, such as by vaccination. Basic biological aspects, such as the development of monoclonal antibodies, will enable the proper characterisation and screening of species and tissues.

By specifically studying growth factors, e.g. hormones and vitamins, it will be possible to identify growth-limiting substances of relevance to whole animal nutrition.

METHODS AND PRELIMINARY RESULTS

This project which is a concerted action commenced in December 1997 and an inventory of methods available in member laboratories has been compiled. The main techniques use explanted tissue fragments that are cultured in commercially available media with the osmolarity increased to facilitate culture of marine invertebrate cells. Tissues being investigated at present include crustacean (Nephrops) gill and hepatopancreas, crustacean (Nephrops and Cancer) hypodermis and molluscan (Ostrea) heart. Specific techniques may be made available on application through the coordinator to the individual member laboratory involved in the specific technique of interest.

A major problem with the culture of all these invertebrate tissues is contamination and a focus of the concerted is to exchange methods on control of pathogenic infection. This is particularly important since it is undesirable to use antibiotics in case these affect the response of cultured cells to controlled infection with pathogens and exposure to toxins in the future.

Another problem being considered is that of slow growth at the ambient temperature for culture of these species (below 15 °C). This means that growth can take
several months before useful numbers of cells can be obtained from explanted fragments. Approaches to increase cell division include consideration of hormones and growth factors that have been identified as useful for insect cell culture.

THE PROPOSED ROLES FOR EACH PARTNER IN THE CONCERTED ACTION

Partner 1, the Dublin Institute of Technology (DIT), will culture Nephrops — hepatopancreas, brain and gills, and coordinate efforts to optimise media for the growth of the cells by all partners.

Partner 2, Heriot-Watt University, will examine the culturing of Peneaus and Nephrops cells, and determine the effect of bacterial pathogens.

Partner 3, the University of Montpellier, will examine cells for validation, and determine the growth of key viruses.

Partner 4, the University of Plymouth, will culture cells from the marine decapod Carcinus, and examine differential gene expression following exposure to heavy metals.

Partner 5, Trinity College, Dublin, will develop systems for culturing sea lice.

Partner 6, University College, Cork, will work with molluscan cell cultures.

Partner 7, the University of León, will apply existing successful fish culture systems to the growth of Peneaus and Nephrops cells.

Partner 8, VESO Ltd, will provide facilities for culturing tissues and the whole animal.

Partner 9, EcoServe Ltd, will measure the effects of organic pollutants on Nephrops cultures.

Partner 10, the Danish Institute for Fisheries Research, will study the effect of bacterial pathogens in cell lines.

Partner 11, the University of Brittany, will examine the role of bivalve cell cultures for ecotoxicology and marine viruses.

Partner 12, the University of Nijmegen, will culture freshwater parasites, e.g. Argulus.

THE PROPOSED WORK OF THE CONCERTED ACTION

1. To coordinate research approaches for the development of reproducible organotypic cell cultures for commercially important aquatic invertebrates, notably Peneaus, Cancer, Ruditapes, Crassostrea and Nephrops spp.

2. To establish standards for the characterisation of the cell types from ‘1’.

3. To coordinate studies to determine the value of these cell cultures and, where relevant, of vertebrate host cultures for the growth of pathogens (bacteria, e.g. Vibrio harveyi), viruses (baculovirus) and parasites (Bonamia, Caligula).

4. To evaluate the cell cultures for use in aquatic ecotoxicology, with emphasis on heavy metals and organophosphorus pesticides.

5. To arrange an international workshop to discuss cell culture techniques for aquatic invertebrates.

6. To produce a state-of-the-art report on aquatic invertebrate cell culture.
FAIR-CT97-3449

Fish pasteurellosis: applied research on vaccine development

OBJECTIVES

The main task of this project is to develop a vaccine(es) against fish pasteurellosis and to establish a vaccination programme that will protect susceptible fish for the duration of their life on the farm. The main research goals of the project are the identification of Pasteurella piscicida antigens which are important for the pathogenesis of fish pasteurellosis, the investigation of the immunogenicity of these products for susceptible fish, the determination of their protective role as vaccine components and, finally, the development of vaccines and vaccination programmes. Through this project and using as a vessel this important bacterial disease of the Mediterranean region of Europe, it is intended to study in depth the immunology of marine fish (other than that of salmonids). This has not yet been researched in detail and the clarification of its various aspects will substantially assist in the future in addressing new disease problems of marine fish species with great economic interest for the European Union.

Contract No: FAIR-CT97-3449
Total cost: EUR 940 155
EC contribution: EUR 879 655
Starting date: 1.1.1998
Duration: 48 months

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**FAIR-CT97-3508**

**Fish cysteine proteinase inhibitors and infectious diseases**

**OBJECTIVES**

The objectives of this project are to isolate and characterise cysteine proteinase inhibitors in fish and study the role of these inhibitors in the defence against bacteria and viruses. Enough primary structure of these inhibitors will be determined either by cloning or direct sequencing for comparison with the mammalian cysteine proteinase inhibitors. The effect and biological mechanism of fish cysteine proteinase inhibitors on apoptosis and other microbiological damage will be investigated. Interesting cysteine proteinase inhibitors will be cloned and expressed as recombinant proteins. These will then be purified and used in biological studies. To investigate the specificity of these inhibitors, cysteine proteinases will be isolated. Our hypothesis is that this ‘inhibitor system’ is a novel non-specific defence system of organisms against infections. The main goal of our project is to elucidate the effects of these proteins and possibly find ways of using these molecules as drugs in aquaculture industries.
5.3. BIOLoGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT97-3640**

*Marteilia refringens* studies: molecular systematics and search for the intermediate hosts of the bivalve mollusc parasites

**SUMMARY**

Since 1968, *Marteilia refringens* has caused serious and recurrent mortalities in the European flat oyster (*Ostrea edulis*) (Alderman, 1979) and continues nowadays to cause high mortalities off the Atlantic coast of France (Grizel, 1985; Anon., 1990).

Under Council Directive 91/67/EEC, this parasite is included in list II of Annex A. This list includes serious pathogens, which cause important losses for the European shellfish aquaculture industry, which should be obligatory declared. An area where flat oysters or mussels are infected with any of these parasites will not be allowed to export to another area free of this disease.

The diagnoses of the two species of *Marteilia* found in Europe (*Marteilia refringens* and *Marteilia maurini*) were carried out using ultrastructural characteristics and host specificity (Grizel et al., 1974; Comps et al., 1982; Figueras and Montes, 1988). However, it is not possible to establish whether one, two, or more species of *Marteilia* exist in bivalve molluscs in Europe. There is an urgent need to set up more sensitive and accurate methods to allow the detection of low prevalence of *Marteilia* and to clarify the taxonomy of this parasite.

The existence of a complex life cycle was postulated early by many authors. Because of the limitations of histology, a sensible and specific tool is needed to screen for *Marteilia* presence in intermediate or alternative hosts.

**OBJECTIVES**

1. Development of molecular tools to clarify the systematics of the European *Marteilia*
   1.1. European *Marteilia* rDNA sequence
      1.1.1. Establishment of the European *Marteilia* rDNA sequences
      1.1.2. Alignment of sequences and taxonomic implications
   1.2. PCR typing of *Marteilia* species/strains
      1.2.1. Design of *Marteilia* specific primers for the PCR
      1.2.2. PCR typing of *Marteilia* species/strains.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

2. Searching for potential Marteilia intermediate hosts using immunoassayand-specific PCR for the parasite

2.1. Marteilia detection in cohabitants using the Claire/Ostrea model

2.2. Marteilia detection in cohabitants using the raft/Mytilus model.

REFERENCES


5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

FAIR-CT97-3643

Evaluation of MHC genes as quantitative trait loci for disease resistance in fish

OBJECTIVES

The objective of this study is to assess the usefulness of highly polymorphic genetic markers, the major histocompatibility complex (MHC) genes, of which it has been firmly established that the molecules they encode are critically involved in the initiation of an adequate immune response to pathogens, as quantitative trait loci (QTL) for improving selective breeding.

The major histocompatibility complex surface glycoproteins play an important role in the immune defence of higher vertebrates where they bind and present endogenous (MHC class I molecules) and exogenous (MHC class II molecules) peptides to T cells, thereby triggering a specific immune reaction towards the pathogen from which the peptides are derived. The exogenous peptides bound by the MHC class II alpha/MHC class II beta molecules are mainly derived from bacteria, whereas the endogenous peptides bound and presented by the MHC class I molecule are derived from intracellular virus or self-proteins. The MHC class I and class II beta genes are highly variable, i.e. there are numerous alleles and numerous haplotypes in a population. Each allele has the ability to bind and present different groups of peptides in more or less successful ways. Thus, the response of an organism towards certain pathogens can be genetically influenced by the MHC haplotype.

The best documented MHC correlation with disease resistance is that in chicken with respect to Marek’s disease where a large number of experimental animals were used. Fish is another example of an animal species where large numbers of offspring can be generated and used in appropriate challenge or selection programmes. Several studies have shown that the MHC is indeed highly polymorphic in fish. Moreover, the number of expressed MHC genes of both classes seems to be limited, making molecular haplotyping of fish a feasible undertaking. This project will be the first to address the possibility to assess the usefulness of MHC genes as QTL for disease in fish, using a large number of experimental animals of which heritability estimates for other parameters have already been established. We have designed this project to look for correlations between particular MHC alleles/haplotypes, and high or low resistance to one bacterial and one viral pathogen in fish. Finding such correlations would enable more specific and therefore more successful breeding programmes in future aquaculture. It would also eliminate the breeding companies’ need to establish and use costly and time-consuming challenge experiments.

The project involves characterisation of both parental and offspring MHC class I, class II alpha and class II beta alleles in a material consisting of 50
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

dams, 50 sires and their 50 pedigrees. Each pedigree is challenged with one bacterial and one (two) viral infectious agent(s) and data are collected on death/survival of each individual. Finally, data on MHC variants and death/survival are tested by statistical analysis to identify positive or negative correlations to disease resistance.

MHC variants in the parental material are more or less characterised and the challenge tests are completed. Remaining tasks are typing of the pedigrees and statistical analysis of the data.

This project mainly focuses on the usefulness of specific DNA markers as QTL for disease resistance. A side effect is the general knowledge generated on MHC alleles and haplotypes in fish. So far, we have identified 21 MHC class II beta alleles in material consisting of 50 dams and 50 sires, which is clearly a large number of alleles for these few individuals originating from one population.

We will shortly finish our analysis on MHC class I and class II alpha variants in the parental material and move on to MHC typing of the pedigrees. The final statistical analysis is expected to begin in the year 2000.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT97-3660**

*Freshwater crayfish and the crayfish plague fungus: disease diagnosis and effects of fungal infection on immunity and reproduction*

**OBJECTIVES**

1. To design molecular tools to detect the crayfish plague fungus.
2. To design tools to identify, follow and trace the origin of infection of crayfish parasites, primarily crayfish plague.
3. To distinguish between resistant and non-resistant populations and individuals with the aim of breeding resistant native European crayfish.
4. To design tools that can be used to determine health status (immune status) of farmed and wild crayfish so that stress conditions can be avoided.
5. To provide knowledge to possibly enhance the efficiency of the immune system.
6. To determine the association between the immune system and reproduction to aid in the development of a better broodstock.

**DESCRIPTION OF WORK AND PROGRESS**

The work to design molecular tools to allow the identification of the crayfish plague fungus is being performed in the laboratory of the associated partner in Munich with continuous contacts with Uppsala University and is well under way. By using information about the internal transcribed regions surrounding the 5.8S nuclear ribosomal gene, it seems to be possible to obtain a sequence within the M region which appears to be specific for crayfish plague strains as judged from several fungi and crayfish plague strains tested. However, more *Astacus astaci* strains need to be tested as well as more closely related species to *A. astaci* than have been tested so far to ascertain that the intended probe will be specific for *A. astaci* strains. So far, however, the results are very promising and it is likely that a crayfish-plague-specific molecular probe that can be used to detect crayfish plague fungus on crayfishes will be produced. The spread of the crayfish plague fungus has been followed in some countries in Europe by using molecular techniques. Thus partner 1 in Uppsala and the university in Munich have been able to show that two recent crayfish plague outbreaks in Germany were caused by fungal strains originating from signal crayfish. By examining DNA from two crayfish plague strains which were isolated from plague outbreaks in two Finnish lakes, we found that one was caused by a strain from signal crayfish suggesting that this crayfish carrying the plague fungus may have been illegally implanted in this Finnish lake. We have also obtained isolates of crayfish plague outbreaks from two different localities in Spain through the courtesy of Dr. Javier Dieguez-Uribeondo and we have now shown that these crayfish plague epidemics are also due to implanted signal
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

The work on identifying different immune factors, which may be involved in mediating resistance towards pathogenic fungi in crayfish, has been dealt with by utilising two completely different techniques. One is based on the fact that the immune genes which have been cloned and characterised from the more resistant crayfish, *Pacifastacus leniusculus*, are similar to those in *A. astacus* and that the use of cDNA probes and DNA/RNA hybridisations is possible. We first tested whether probes for the two immune genes prophenoloxidase and the glucan binding protein, BGBP, hybridised with RNA from the noble crayfish, *A. astacus*. In both cases we found that the probes developed in signal crayfish recognised corresponding RNA in the noble crayfish. However, since individual variation in gene expression of the tested genes during an infection was observed, we decided instead to use RNAase protection analysis of the transcripts, which is a more sensitive assay than a Northern blotting analysis and permits analysis of individual animals. This methodology is presently being optimised in the laboratory of partner 1.

During an infection with the crayfish plague fungus in noble crayfish, a rather dramatic effect on the haemocyte behaviour was observed, since the haemocytes were shown to be strongly activated at early stages of infection and at later stages the haemocyte number was lowered quite significantly. This means that the animal has few or small options to combat the ongoing infection and will die as a result of this experimental fungal infection. The other technique to identify changes in immune gene expression and to possibly identify new genes, which are affected in their expression during an infection, is by using a differential display RT-PCR technique. For this purpose RNA has been isolated from crayfish-plague-infected and healthy *A. astacus*, respectively. In signal crayfish total RNA was isolated from crayfish previously injected with bacteria and from non-injected signal crayfish. The display from the experiments with signal crayfish gave two transcripts which were induced following injection with bacteria and two which were downregulated. The resulting PCR products were subcloned but since the PCR products were located in the UTR (in the so-called untranslated region) no sequence comparisons could be made and instead we concentrated our experiments on the noble crayfish. In this crayfish two bands were found to be induced and two were decreased in their expression following a fungal infection. These products are now being subcloned and sequenced in the laboratory of partner 1. Partners 1 and 3 have analysed the effects of crayfish plague fungus on lipoproteins, lipid transport and lipid accumulation in eggs during reproduction in crayfish. Two experiments were performed to provide initial and detailed data on haemolymph lipids, lipid classes, and fatty acid distribution in two crayfish species before and after experimentally induced fungal infections. The data obtained from pooled samples in experiment 1 showed that phospholipids (PLs) are the most abundant lipids in the haemolymph and that triacylglycerols (TAGs) were detected only in females. Fungal infection reduced the levels of lipid classes (PLs, diacylglycerols (DAGs) and cholesterol) but had no effect on the distribution of fatty acid in samples collected from male and female crayfish of the two tested species (*A. astacus* and *P. leniusculus*). Similar results were found in the HDL and VHDL fractions obtained from the haemolymph. Preliminary results indicated some effect on the integrity of the haemolymph lipoproteins after fungal infection as revealed by polyacrylamide gel electrophoresis. Moreover, the effects in *A. astacus* were more pronounced than those for the more resistant *P. leniusculus*. In order to verify and estimate the individual variation and assess the effect of fungal infection during the reproductive season in vitellogenic females, a second experiment was performed using *A. astacus*. Results from this experiment indicated that the effect of repeated bleeding of crayfish was larger than the effect obtained following fungal infection probably as a result of the relatively high variation between samples within each treatment group.

ACHIEVEMENTS

The possibility to produce a specific probe to detect the crayfish plague fungus on crayfishes is at hand, but still needs extensive additional work before it can be evaluated for its suitability as a molecular probe to detect this pathogenic fun-
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

Epidemiological studies have been carried out using an RAPD-PCR technique and thus partners 1 and 2 have shown that signal crayfish have caused crayfish plague outbreaks in both Germany and Finland. Using this method, we have developed a simple tool to use in such epidemiological studies since we have characterised about 30 different *A. astaci* strains and found that they can be placed in four different genotypes and this allows us to determine the origin of the crayfish plague fungus from any infected lake or water body. Two papers have been published in international journals. One lipoprotein involved in clotting has been cloned and characterised and found to belong to the vitellogenin superfamily of proteins which is of considerable interest since this shows that reproduction and an innate immune reaction are linked. A manuscript on the characterisation of this clotting protein is in press in *Proc. Natl. Acad. Sci. USA*. The work on studying changes in gene expression during a fungal infection has so far shown that some transcripts are upregulated and some are downregulated. These transcripts are presently being cloned and sequenced and we anticipate that during the second year we will have these genes characterised. Partner 3 has also successfully characterised the lipid classes and fatty acid distribution in two different crayfish species and an interesting finding was that triacylglycerols are only present in females.

**FUTURE ACTIONS**

Partner 2 in collaboration with partner 1 will continue with their work on designing and testing a molecular probe to be used to detect the crayfish plague fungus on different crayfish species. Partner 2 will continue to characterise the genes which are up- and downregulated and also with the new method of RNAase protection analysis study if more genes are affected by a fungal infection during different periods after infection. Partners 2 and 3 will continue their work on the study of changes in amount of lipids and lipoproteins during a fungal infection.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT97-3691**

**Effects of environmental stressors on antimicrobial activity in marine bivalve molluscs (ESAM)**

**OBJECTIVES**

The overall objectives of the project are to characterise the immune cells of the developmental stages of the mussel *Mytilus edulis* (galloprovincialis) and to develop nucleotide probes to antimicrobial peptides for investigating the expression of these antimicrobial peptides in the developmental stages of *Mytilus*. The project will also quantify the susceptibility of larvae to specific bacterial pathogens. The effects of environmental stressors on humoral and cellular immune function in developmental stages will be investigated and the cell/tissue specificity of the stressors identified. Finally, the project will establish whether environmental factors (temperature, specific environmental contaminants) increase the susceptibility of larvae to specific pathogens.

**DESCRIPTION OF WORK**

Characterisation of the immune cells of the developmental stages of *Mytilus* will involve light and electron microscopy, and immunocytochemistry, together with assays for phagocytosis and release of reactive oxygen metabolites. The development of nucleotide probes to antimicrobial peptides will involve total RNA extraction and electrophoresis and probe constructions according to the amino acid sequences. Northern blotting validation will be carried out on untreated adults and detection of transcripts will be carried out by reverse-transcription PCR. The susceptibility of larvae to specific bacterial pathogens will be undertaken using laboratory exposure to specific concentrations of bacteria. The effects of environmental stressors on humoral and cellular immune function in developmental stages will also involve laboratory exposures. It is proposed that for all the tasks outlined the following developmental stages will be investigated:

- trophophore larvae — non-shelled ciliated planktonic stage (approximately 24–48 hours post-fertilisation);
- veliger larvae — shelled planktonic stage, actively swims and has functional gut (approximately one to four weeks post-fertilisation);
- postlarval stages — successfully completed settlement and metamorphosis (> six weeks post-fertilisation).

**STATE OF PROGRESS**

Immunocytochemical labelling with antibodies to adult haemocytes, total actin and *Mytilus* fibronectin has been carried out on larval and postlarval
mussels. Biochemical characterisation of actin and fibronectin in larval and postlarval mussels has been carried out. The release of reactive oxygen metabolites by disaggregated trochophore and veliger larvae has been investigated. Nucleotide probes for the defence peptides, MGD-1, mitilin, myticin and 18S-ribosomal probe have been constructed and used to detect antimicrobial peptides in *M. edulis* at different developmental stages. Protocols for determining and quantifying the viability of larvae have been investigated using both staining and morphological criteria. Protocols for investigating the effects of bacterial pathogens on mussel larvae have been investigated.

**ACHIEVEMENTS**

The results showed that actin from larvae and postlarvae was separated by SDS-PAGE and the Western immunoblot was labelled with anti-total actin, with the antibody recognising a 43 kDa protein band. The Western blotting analysis of larval and postlarval FN showed labelling with autologous anti-FN antiserum with a 220 kDa band, which was coincident with the adult *Mytilus* haemolymph FN used as standard. Immunocytochemical labelling identified sites for actin and fibronectin in larvae and postlarvae but failed to detect adult haemocyte antigens in the larvae. The results for release of oxyradicals showed for the NBT assay that PMA had an inhibitory effect on larval cells from both the trochophore and veliger stages. LPS did appear to stimulate larval cells. With the cytochrome c assay, PMA and PLS increased the superoxide generation in the larval cells. All individual mussels expressed mytilin and myticin genes whereas only three out of eight expressed the MGD-1 gene. There was no hybridisation signal on RNAs from eggs and trochophore larvae, strongly suggesting the absence of antimicrobial gene expression during the first stages of development. RNAs from postlarvae as small as 1 mm showed a hybridisation signal with mytilin B and myticin A probes, within the same size range as those of the adults. Of the staining techniques used for assessing larval viability, only fluorescein diacetate was suitable. In general, it was found that morphological criteria were easier to use, allowing fixation and consequently more accurate detection of abnormalities.

**FUTURE ACTIONS**

Research on the susceptibility of the developmental stages to specific bacterial pathogens will continue. Work to investigate the effects of environmental stressors on humoral and cellular immune function in the developmental stages of the larvae, and to identify the cell/tissue specificity of the stressors will begin. The aim is to establish whether environmental factors (temperature, specific environmental contaminants) increase the susceptibility of larvae to specific pathogens.
5.3.2. Health of aquacultured species

FAIR-CT97-3760

Concerted action workshop on minimum inhibitory concentrations of antimicrobials against fish pathogenic bacteria

OBJECTIVES

The main objective is to bring together, by means of a concerted action workshop, the major Community laboratories (plus a limited number from outside Europe) working on aspects of the use of chemotherapeutants, particularly antibiotics and the problem of drug resistance in aquaculture. The primary theme of the workshop will be to enable these experts to re-examine this problem and, in particular, to discuss and gain agreement on recommendations for a uniform method of determining minimum inhibitory concentrations (MICs) for the most important antimicrobials against the most important fish pathogens. The intention is that workshop participants should present these recommendations to the Commission and publish them, together with the proceedings of the workshop, in a peer-reviewed international journal (Aquaculture).

The above objectives comply with the objectives of the FAIR work programme under a number of headings, including 5.2.2 ‘The effects of aquaculture on the environment’. A more standard MIC methodology should assist in reducing the inappropriate use of antibiotics to control bacterial disease and thereby reduce the adverse effects of the use of medicines in aquaculture on the environment.

Additionally, the proposed workshop falls under 5.3.2 ‘Health of aquacultured species’, in particular the substitution of an optimisation of therapeutics. The objective of this workshop is to arrive at an agreed method for MIC determination, which will itself directly provide for the proper selection of the correct therapeutic dose of the most appropriate antimicrobial agent for the bacterial pathogen concerned.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT97-3963**

*Cataracts in farmed fish — A multidisciplinary initiative for scientific progress*

**OBJECTIVES**

Cataracts (reversible or irreversible lens opacities) are a production disorder of several species of fish. The aim of this project is to disseminate knowledge on occurrence and causation of cataracts in farmed fish, and to initiate further research to prevent and control the disease. To achieve this goal, we propose to create a multidisciplinary network of European scientists and aquaculture industrialists to overcome present restraints in research. The project will thus contribute to securing the health and well-being of farmed fish, and to improving the cost-effectiveness of European aquaculture. The competitive advantage of European suppliers of materials or services to the international aquaculture market will be increased. Successful control of cataracts is considered even more important in order not to compromise consumer perception of the aquaculture industry as such, particularly regarding its ethical standards of production and the quality of its products. These issues are of major importance for the industry’s further competitiveness in the international food market. Bringing the aquaculture industry, fish pathologists and nutritionists together with multidisciplinary working ophthalmologists, a new and unique scientific collaboration for the benefit of future aquaculture production, fish welfare and fish health research will be created; in itself, a most valuable goal.

These main objectives have been detailed as follows:

1. the project will disseminate scientific knowledge and state of the art in cataract research, and discuss current and planned work conducted in this field;
2. the project will facilitate exchange of study specimens and transfer of research methodology between laboratories;
3. the project will initiate epidemiological studies and thus provide new scientific data on the occurrence and economy of the disease;
4. new research on the physiology of the fish eye and the pathogenesis of lens cataracts will be stimulated;
5. financial support for explanatory studies on the cataract problem will be sought.

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**EC contribution:** EUR 190 000  
**Starting date:** 1.1.1998  
**Duration:** 36 months

**COORDINATOR**

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5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE
5.3.2. Health of aquacultured species

FAIR-CT98-4003

DNA vaccines for aquaculture: development and testing of plasmid vectors for vaccination against bacterial and viral fish pathogens

OBJECTIVES

The global objective is to further the development of DNA vaccine against fish pathogens for use by the aquaculture industry. The specific objectives are:

1. To produce and test many different genes from vibriosis for their protective efficacy within DNA vaccines; this information will aid the development of DNA vaccines against many bacterial diseases;
2. To develop a DNA vaccine against bacterial kidney disease (BKD), a serious emerging disease for which there is currently no effective vaccine;
3. To develop methods to administer DNA vaccines by dip, immersion or oral delivery;
4. To evaluate the role of CpG immunostimulatory sequences for augmenting immune responses to DNA vaccines and develop CpG-optimised DNA vaccine vectors;
5. To measure the kinetics of humoral and cell-mediated specific and non-specific immune responses to DNA vaccines and CpG motifs by use a cannulation system that allows repeated blood sampling from individual fish;
6. To determine certain safety aspects of DNA vaccines including the kinetics and longevity of antigen expression, the persistence of plasmid in the vaccinated host, and the risk of integration in the vaccinated fish.
FAIR-CT98-4026

Short- and long-term effects of genetic immunisation on the fish immune system

OBJECTIVES

Nucleic acid immunisation can induce antibodies, cytotoxic T lymphocytes (CTL) and protection against many pathogens. The underlying mechanisms remain obscure and must be understood to permit rational manipulation and optimisation of the methodology.

Using the rainbow trout (RBT) and viral haemorrhagic septicaemia virus (VHSV) genes as a model of immunisation, the aims of the present project are:

1. to analyse the effects of a long-lasting antigen production on the specific and non-specific immune response;
2. to analyse the effect of the continuous delivery of the antigen on the shape of the B and T cell repertoires;
3. to evaluate the modulating potential of selected cytokine or virus-induced genes on the immune response to genetic immunisation.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT98-4036**

*Nodavirus disease in cultured marine fish in Europe*

**OBJECTIVES**

The objectives of the project are to study nodavirus disease in cultured fish through characterisation studies of nodavirus, comparative assessment of new and established diagnostic methods, experimental disease transmission trials and evaluation of the fish immune response to infection. The project should allow development of practical, effective and reliable methods for the diagnosis, prevention and control of nodavirus infections of cultured sea bass and other marine fish in Europe and other Mediterranean countries. Because the project deals with a disease which is of major importance to the aquaculture industry, especially in Italy and Greece, an industry representative from these countries, proposed by the Federation of European Aquaculture Producers (FEAP), will participate in the project as an expert consultant directly linked to the coordinator. One official of the FEAP will also be invited once a year to the meetings.

**METHODOLOGY AND RESEARCH TASKS**

The methodology will consist of making a collection of nodavirus strains originating from a broad geographical range in the Mediterranean region and also from other parts of the world. This virus collection will be studied by standard virological techniques for the purpose of virus characterisation and to investigate the sensitivity of nodavirus to a variety of physical and chemical treatments. The antigenic and genetic relationships of nodavirus strains from the Mediterranean and other geographical regions will be examined to evaluate the specificity of different diagnostic procedures and to try to correlate the variations with the host fish species and/or the geographical origin. The virus collection will be used for a comparative assessment of existing disease diagnostic methods to evaluate the relative merits, limitations and applications of these alternative procedures. When needed, studies for improving the methods will be initiated. Experimental disease transmission trials will be performed in larval, juvenile and adult stages of fish to determine: (i) possible routes of nodavirus infection; (ii) the persistence of infection in fish tissue; and (iii) the development of the carrier state in clinically unaffected fish. Finally, immunological studies will be conducted in order to elucidate the humoral immune response of fish to natural infection and to the inactivated virus in order to provide useful information for a future vaccination control strategy.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE
5.3.2. Health of aquacultured species

**FAIR-CT98-4064**

*Diagnostic methods and reference panel of reagents for detection and typing of fish viruses*

**OBJECTIVES**

The aims of the proposed study are:

1. to improve and harmonise research and development of biotechnological diagnostic procedures for viral diseases in aquacultured fish;
2. to investigate the genetic diversity among fish rhabdoviruses;
3. to establish an international database for available diagnostic reagents related to viral diseases in aquacultured fish.

Specific objectives are:

1. to produce and characterise virus-specific monoclonal antibodies for diagnostic purposes;
2. to develop DNA probes and primers for hybridisation and PCR assays for detection and typing of viruses;
3. to generate an international database describing available reagents for diagnosis of fish viruses;
4. to perform RNAase protection assays and phylogenetic analyses of the fish pathogenic rhabdoviruses VHSV, IHNV, SVCV and ELTV in order to determine the extent of diversity among the genomic variants of the respective viruses;
5. to develop diagnostic kits for commercial use.

**DISSEMINATION ACTIVITIES**

A workshop will be organised for the evaluation, and standardisation of diagnostic methods in fish virus diagnostics in Europe.

Results will be published by submissions to international scientific journals and by presentations at scientific meetings.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

FAIR-CT98-4087

DNA vaccination of aquaculture fish with emphasis on VHSV in rainbow trout as a model

OBJECTIVES

The proposed project concerns the development of DNA vaccines for application in farmed fish using VHS virus infection in rainbow trout as a model. Focus will be on the development of vaccine formulations that provide high efficacy by the different routes of administration currently used in aquaculture. Assessment of vaccine safety is also included.

The objectives of the project are:

1. to prepare and test DNA vaccine constructs;
2. to identify and develop an optimal formulation for delivery of a DNA vaccine by injection and immersion in rainbow trout using VHS as a model;
3. to perform a limited characterisation of the humoral and cellular immune response following DNA vaccination against VHS compile data;
4. to present a summary safety report in cooperation with projects FAIR-PL98-4003 and 4026.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT98-4129**

*Environmental factors and shellfish diseases*

**OBJECTIVES**

This project aims to:

1. provide a better understanding of bivalve-pathogen interactions;
2. identify environmental factors that modulate shellfish internal defence by using biological models developed by the partners;
3. use the above information for prediction and management of the impact of disease on cultivated stocks.

As the stress generated by acute or long-term changes in environmental factors may have an effect on the interaction between hosts and pathogens in shellfish populations, the following fundamental questions will be addressed:

- Are the seasonal or occasional changes in infection or parasitism levels which are actually observed in cultured stocks related to measurable changes in host defence mechanisms?
- Which mechanisms in the defence process are targets for environmental immunosuppressors?
- Could the measurement of individual defence capacities become a tool for risk assessment and stock management?

**METHODOLOGIES AND RESEARCH TASKS**

Two models will be studied in parallel since previous work by the partners suggests that these models will provide important information on complementary steps in the pathogenic process. The bonamiosis model (*Ostrea edulis/Bonamia ostreae* — Oe/Bo) involves an internal parasite (Bo) and current investigations are focused on host (Oe) susceptibility and specificity at the cellular level. Vibriosis (*Ruditapes philippinarum/Vibrio tapetis* — Rp/Vt) is a true infectious disease which is clearly influenced by environmental factors and well adapted to epizootic monitoring. Each model provides an experimental system for investigation of a range of biological processes, from the overall susceptibility of individuals to immune cellular responses in haemolymph.
5.3.2. Health of aquacultured species

**FAIR-CT98-4217**

**FISCI: a new bio-index for the assessment of stress condition in aquacultured marine fish**

**OBJECTIVES**

The overall objective of the project is to develop a non-invasive method for detecting general and specific stressor-related stress effects in fish reared in aquaculture. Within the general research framework, the project aims at two interrelated objectives:

1. To detect, analyse and quantify stress effects, under both laboratory and fish farm conditions, in two Mediterranean marine fish species, gilthead sea bream (*Sparus auratus*) and European sea bass (*Dicentrarchus labrax*), which are of major economic importance for European aquaculture, using as parameters changes in the skin and gill epithelia, and the mucus layer covering the skin: (i) changes in tissue morphology; (ii) enzymatic activity in skin and skin mucus; (iii) concentrations of the stress hormone cortisol in blood serum and skin mucus; and (iv) non-enzymatic biochemical parameters in skin mucus samples. The stressors to be applied (low dissolved oxygen levels, high ammonia levels, and handling stress) are relevant to aquaculture.

2. To select subsequently the analytical methods and stress parameters that will prove to be reliable and easy to perform, using a sampling procedure from which the sampled fish can easily recover. The selected methods will be combined as bio-indicators through the establishment of a new stress index, the ‘fish stress condition index’ (FISCI), that will enable the characterisation of stress condition in cultured fish and will reflect both fish health and water quality. This will provide fish farmers with a protocol method to check the stress condition of their fish.

**Contract No:** FAIR-CT98-4217  
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**EC contribution:** EUR 670 000  
**Starting date:** 4.1.1999  
**Duration:** 36 months

**COORDINATOR**

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5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE
5.3.2. Health of aquacultured species

FAIR-CT98-4334

Diagnosis of oyster herpes-like viruses: development and validation of molecular, immunological and cellular tools

OBJECTIVES

The aim of the project is to develop specific tools for diagnosing herpes-like virus infections in oysters and to validate these reagents by using them in different European laboratories involved in bivalve epidemiological surveys. This will be done using techniques developed by the partners to characterise viruses by studying their genomes and immunologically reactive proteins, to cultivate oyster cells and vertebrate cell lines and to perform epidemiological surveys among bivalves. The project’s objectives are:

1. to obtain the complete oyster herpes-like virus DNA sequence with determination of the genome structure;
2. to compare oyster herpes-like viruses with other viruses belonging to the Herpesviridae family on the basis of sequence data and genome structure;
3. to develop molecular tools for oyster herpes-like virus detection using the complete virus DNA sequence data;
4. to develop immunological tools for oyster herpes-like virus detection;
5. to develop cellular tools for oyster herpes-like virus detection using oyster primary cell cultures and vertebrate cell lines;
6. to apply developed diagnostic tools for herpes-like virus detection in oyster samples from different geographical locations.

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EC contribution: EUR 649 738
Starting date: 4.1.1999
Duration: 36 months

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5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.2. Health of aquacultured species

**FAIR-CT98-4398**

*Infectious fish rhabdovirus from cloned cDNA: a tool for salmonid protection in aquaculture*

**OBJECTIVES**

The goal of this project is to transpose to the fish rhabdovirus the reverse genetic technology that has been used with success for mammalian negative-strand RNA virus like rabies and the vesicular stomatitis virus VSV to generate new live vaccines which are genetically tagged and cost-effective, deliverable by bath immersion and safe for other fish species.

The main objectives for the project are as follows:

1. Construction of a fully attenuated IHNV strain following targeted mutations:
   - generation of a full-length cDNA IHNV genome (12 Kbases) as well as individual nucleoprotein, phosphoprotein and polymerase encoding cDNA;
   - obtaining new infectious IHNV virions following co-transfection and T7-driven vaccinia virus infection in fish cell lines.
2. Creation of a new viral vaccine strain expressing IHN/VHS glycoproteins:
   - explore the feasibility of the insertion of the VHSV glycoprotein encoding gene in the IHN genome using as a potential insertion site a non-essential gene (Nv);
   - evaluation of the efficacy of that new bivalent live vaccine.

As a first step we have chosen to apply on IHN the reverse genetic technology to produce a safe live vaccine but also to develop a potentially very powerful bivalent live vaccine. If successful, this programme may be the first step towards a new generation of fish vaccines of improved efficacy against rhabdovirus disease affecting aquacultured fishes (spring viremia of carp, SVC, perch rhabdovirus disease, PRD, etc.) but which can also be used to fight other viral diseases such as IPNV using the rhabdovirus as the gene vector.

The improvement in fish protection will play a role in improving the health of aquacultured species and consequently the productivity of fish farms. Thus, this programme will have a beneficial socioeconomic impact, in particular for small-scale fisheries where productivity and profitability are crucial survival conditions.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.3. New species

**FAIR-CT95-0407**

Common dentex, a prime new species for aquaculture

Development of methods for reliable egg production

**OBJECTIVES**

The overall aim of the project is to provide a scientific and technical basis for effective and reliable reproduction of the common dentex (*Dentex dentex*), a prime new species for intensive fish farming. The main objectives of the project are:

1. to provide basic knowledge on the reproductive biology of this species;
2. to investigate external factors likely to be used for manipulating the spawning period;
3. to develop an appropriate and cost-effective methodology for synchronisation of spawning and increasing yields and quality of gametes;
4. to support the development of a new candidate species for aquaculture.

**INTRODUCTION**

*D. dentex* is a highly valued table fish in the Mediterranean region and elsewhere in the tropics and the continuously increasing catches suggest a high and unsatisfied demand for this sparid fish. Rearing experiments with juveniles have identified *D. dentex* as highly suitable for intensive aquaculture (Bibiloni et al., 1993; Efthimiou et al., 1994). However, studies on this species are sparse (Bauchot and Hureau, 1986; Franicevic, 1991; Kentouri et al., 1992; Morales-Nin and Moranta, 1997) and basic knowledge of the reproductive biology under intensive farmed conditions is extremely limited and conflicting. D’Ancona (1950) concluded that *D. dentex* is a gonochoristic sparid. However, Bauchot and Hureau (1986) and Glamuzina et al. (1989) stated that *D. dentex* was a protandric hermaphrodite, with sex reversal unlikely to be obligatory. A recent field study (Morales-Nin and Moranta, 1997) supports the initial information of *D. dentex* as a gonochoristic species.

The project comprises four major tasks: (i) morphological, histological, endocrine and metabolic study of female and male sexual cycles in fish aged 3 months to 47 months (which covers three spawning seasons; (ii) investigation of the role of melatonin in conveying important information about diurnal and annual changes in day length and investigation of the role of photoperiod on the control of reproduction for out-of-season egg production; (iii) spawning induction treatment and identification of the steroids synthesised by the gonads, as well as identification and measurement of the maturation-inducing steroid(s) or its (their) metabolites in the water as a candidate marker for monitoring spawning success; (iv) investigation on the
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.3. New species

importance of the sex ratio and interaction within sexes on the efficiency of spawning. Tasks are supported by quantitative and qualitative analysis of released eggs and sperm.

RESULTS

Data have already been obtained on the timing of sex differentiation, sexuality pattern, and gametogenetic cyclicity.

Sex differentiation in _D. dentex_, occurs between 5 and 12 months of age. Among the 323 individuals studied so far, no bisexual gonads (apart from one male with a few scattered previtellogenic oocytes in its testes) nor any other suggestion of sexual inversion were found. The relative frequency of both sexes (sum of all studied cohorts) did not differ significant from 50 %, although variations as a function of the age and the cohort of the fish were observed. Five stages of maturity have been determined for females (F1: beginning of ovogenesis; F2: previtellogenesis; F3: endogenous vitellogenesis; F4: exogenous vitellogenesis; F5: final maturation) and six for males (M1: beginning of spermatogenesis; M2: full spermatogenesis; M3: completed spermatogenesis; M4: full maturity; M5: post-spawning; M6: beginning of the second spermatogenesis). From the end of March to the end of May, about 100 % of the males, older than one year, are mature and give sperm (stage M4), whatever they are, 21–23, 33–35 or 46–47 months old. Young males, 10–12 months old, develop testes, but these are small (GSI lower than 0.1 %) and do not produce sperm. The females, older than one year, are mature nearly simultaneously with the males. However, the percentage of mature females is a function of the age: 30–70 % at 21–23 months, 50–80 % at 34–35 months and 70–100 % at 46–47 months. The spawning period (Crete, 35 ºN) takes place in spring (end of March until the end of May/start of June) at a temperature range of 15.4 to 21.4 ºC.

Photoperiod appears to be the main environmental factor controlling spawning time. Fish exposed to a 12-month-long seasonal photoperiod cycle advanced by three months in relation to the natural cycle (Group A) and at an almost constant water temperature (18.5–20.5 ºC) spawned almost three months in advance than controls (Group B). Duration of the spawning period for Group A fish was 65–80 days (6 January to 3 April 1997) and for controls 66–72 days (26 March to 5 June 1997). No difference in the average number of released eggs, fecundity, proportion of viable/non-viable eggs, and semen characteristics was found among the experimental groups. Concerning melatonin, results indicate a secretion profile closely resembling those in other fish species studied to date, with levels which rise immediately at the onset of darkness and fall to basal at lights on.

Seasonal fluctuations and maturity-stage-dependent differences were found in almost all of the estimated serum components. Amongst 180 males, only three of them showed detectable serum vitellogenin levels. No vitellogenin was detected in undifferentiated fish and in females at stage F1. In females vitellogenin was mainly detectable in stages F4 and F5. Mean levels increased from stage F3 (52.6 ± 22.7 µg/ml) to F5 (1 124.2 ± 170.3 µg/ml) whatever the age. In males the maximal plasma concentrations of the androgens 11-ketotestosterone (11-KT) and testosterone (T) were 3.61 ± 1.19 ng/ml and 4.04 ± 1.19 ng/ml respectively. In females maximal plasma concentrations of T and in 17ß-oestradiol (E2) were 1.21 ± 0.28 ng/ml and 0.75 ± 0.16 ng/ml respectively. Concerning the seasonal cycle of T and E2 in females and T and 11-KT in males, levels were undetectable or near the detection limits of the assay from June to December with levels rising to a peak during March or April (spawning period). In females serum thyroid hormones (T3, T4), total proteins (TP), total lipids (TL), cholesterol (Chol) and triglycerides (Trig) displayed minimum values in immature fish (F2, F3) and maximum in mature fish (F4, F5). In males no significant differences were observed in T3 serum levels among the maturity stages, while the rest of the determined components showed maximal values in stage M4 (Trig in M3). Glucose, in both sexes, did not differ significantly among maturity stages.

The energy content of the female gonads was positively correlated with the maturity stage, having a maximum value at stage F5. The energy density of female gonads varies between about 22 (F2) and 27 kJg⁻¹ (dw) (F5). The energy density
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.3. New species

of the male gonads had no clear correlation with the maturity stage or season. The energy densities of liver and muscle varied greatly between individuals. There seems to be a slight decrease in the energy density prior to the spawning period. Total energy (TE) contents of the liver increased with increasing gonadosomatic index until it decreased significantly some months before the spawning period. TE of the gonads follows the development of GSI. Lipid densities (LDs) of the liver and gonads exceed that of muscle. Glycogen densities (GDs) of muscle and gonads are extremely low if compared with the GD of liver. In liver the GD seems to follow the reproduction cycle of D. dentex. The decrease both in the energy density of muscle and liver and total energy content of the liver may indicate the transfer of energy from somatic tissue to female gonads during the period of fast gonadal development of female fish. Statistical analysis on the correlation between energy densities, total energy contents, lipid density and glycogen density is in progress. Analyses of the fatty acid content of gonads, liver and muscles are in progress. Preliminary data showed that the main polyunsaturated fatty acids (PUFAs) of the samples analysed so far are docosahexaenoic (DHA, 22:6n-3) and eicosapentaenoic (EPA, 20:5n-3) acids. The main n-6 PUFAs were linoleic (18:2n-6) and arachidonic (20:4n-6) acids in TG and PL fractions, respectively. Palmitic (16:0) and oleic (18:1n-9) acids are the main fatty acids of the saturated FA and monoenes, respectively.

A study on the identification of MIS is in progress. The initial results indicate that a D. dentex ovary has the potential for producing both 17,20-dihydroxy-4-pregnen-3-one and 17,20,21-trihydroxy-4-pregnen-3-one, the latter being hardly detected. Steroid levels in water were comparatively higher than in plasmas. The trend for each steroid for a particular treatment group was similar whether it was the free, sulphated or glucuronidated steroid. In addition, amounts of steroids within a group followed a similar trend, with levels of 5β-pregnane-3α,17,20β-triol (5β 3α > 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) > 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β, 21-P). Regardless of treatment, in all groups the amounts of steroid followed the general trend glucuronidated > sulphated > free.

DISCUSSION

The present study describes for the first time details on the reproductive biology of D. dentex reared in intensive culture conditions. Results indicate that although some (young) specimens could have bisexual gonads and the sex ratio could in some cohorts differ significantly from 50 % due to some undetermined reasons, the common dentex is a gonochoric fish. Both sexes attained full maturity in their second year of life (21–23 months old); however, the percentage of mature females is a function of the age. The spawning period takes place in spring (end of March to beginning of June) and photoperiod manipulations can lead to out-of-season egg production. Spawning can also be induced by using GnRHa implants (in the form of EVAC pellets or microspheres) with results similar to those reported for another farmed species, the common sea bream Pagrus pagrus (Fostier et al., 1996). Initial data on the identification of MIS showed that probably 17,20β-P or 17,20β, 21-P is the MIS in D. dentex and an intensive metabolism seems to occur, thus MIS production could be transient. It is expected that completion of all running experiments during the third year of the project will provide a basic understanding of the reproductive biology of the common dentex and will support the development of a new candidate species for aquaculture.

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REFERENCES


FAIR-CT96-1623

Biology of sea urchins under intensive cultivation (closed-cycle echiniculture)

OBJECTIVES

The ultimate objective of the project is to control every life stage of the most valuable species of European edible sea urchins (*Paracentrotus lividus* and *Strongylocentrotus droebachiensis*) under intensive cultivation (closed-cycle echiniculture) to produce high-quality gonads (roe, i.e. the edible part of the animal) at a pilot scale. The obstacles that prevent the intensification of echiniculture have been clearly identified: post-settlement survival and growth rate need to be improved, and the carrying capacity of the rearing structures needs to be increased by bypassing the main limiting factors, i.e. depletion in dissolved carbonates and accumulation of carbonic acid. Moreover, the quality control of gonads and optimisation of gonad growth are key factors that have to be addressed. The proposed work aims to investigate aspects of the biology of cultivated sea urchins related to these obstacles, to finalise technical enhancements of the cultivation procedure in either eliminating or bypassing these obstacles, and to adapt the rearing method presently used for *Paracentrotus lividus* to *Strongylocentrotus droebachiensis*.

The sea urchin cultivation procedure uses special devices constituted by several superposed shallow tanks. The shallow tanks, namely the so-called toboggans, hang over a reserve tank where water is treated before being reused. This treatment includes aeration, decantation and thermoregulation. A centrifugal pump transfers water from the reserve tank to the top level, and the water then recirculates thanks to gravity from one level to the other. Water renewal is regulated between 250 and 600 % of the total volume per day following the population density. In this semi-intensive cultivation procedure, the biomass is maintained below 5–6 kg of sea urchins larger than 10 mm diameter per m² of toboggan (growth structure) or between 0.2 and 1 kg of sea urchins smaller than 10 mm per m² of toboggan (pre-growth structure). The control of the whole life cycle of sea urchins in closed-cycle cultivation aiming to obtain adult individuals with high gonadal productivity was shown to be possible in such installations. A method for production of large individuals — namely the standard rearing method (SRM) — was already perfected, though some basic aspects clearly need to be improved. These aspects have been previously identified and will be the subject of the investigations detailed below.

**Task 1:** To improve early postmetamorphics’ survival and growth rate by investigating the improvement of feeding of precompetent and competent larvae and start-feeding routines of early postmetamorphics, and determining the optimal biochemical composition of the proposed food.

**Methodology:** The food given to the larvae (microalgae) and the early juveniles (biofilm and macroalgal plantlets) will be controlled by biochemical
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5.3.3. New species

The use of microalgae previously enriched in different lipids, sterols and amino acids will be investigated. These enriched foods will be tested and their impact on the development and survival rate of larvae and postmetamorphics will be measured.

Task 2: To increase the optimal biomass of adult sea urchins in the cultivation structures by controlling factors which limit the biological carrying capacity of the physical cultivation environment. These limiting factors have already been identified: the depletion in dissolved carbonates which are incorporated into the skeletons of the sea urchins during somatic growth, and the accumulation of carbonic acid produced both by respiration and skeletogenesis. 

Methodology: The somatic growth of postmetamorphics under various alkalinity conditions will be measured to precisely determine the variations of alkalinity tolerated by cultivated postmetamorphics. The dissolved inorganic carbon (DIC) cycle in a cultivation structure for different biomasses will also be studied. The data collected will be integrated into the mathematical model to take the carbonate factor into account in the calculations (see task 5) and to evaluate the potential impact of a particular filter used to stabilise alkalinity in intensive cultivation.

Task 3: To develop an objective quantitative method for quality control, and to investigate the effect of enrichment of artificial foods (i.e. pellets) on gonad quality.

Methodology: Near infrared spectroscopy (NIR) is a fast method of measuring the quality of seafood, but precise calibration is required with traditional methods: biochemical and histological standards and calorimetric methods.

Task 4: To study the bioecological differences between *Paracentrotus lividus* and *Strongylocentrotus droebachiensis* reared in similar cultivation systems and consequently adapt the cultivation method.

Methodology: The production of *Strongylocentrotus droebachiensis* will be upscaled from laboratory scale to pilot scale adapting the methodology already successfully used for *Paracentrotus lividus*. Passive integrated transponder (PIT) tags will be used for the individual identification of broodstock animals. This is a new methodology for tagging sea urchins that has been recently developed. It is the only practical alternative to physical isolation of individual broodstock animals.

Task 5: To update an existing mathematical model of cultivated sea urchins’ productivity with information gathered during this project; to perform a forecast microeconomic analysis of an echiniculture activity.

Methodology: Computer modelling of the production cycle involves calibration, modification and expansion of an existing model developed by partner 1. The model will be expanded to include the survival of early postmetamorphics, the gonads’ quality and the effect of alkalinity on somatic growth and will also be calibrated for *Strongylocentrotus droebachiensis*. 

FAIR-CT96-1623
**FAIR-CT97-3544**

A multidisciplinary evaluation and optimisation of the production characteristics of different strains of commercially cultured flatfish

**OBJECTIVES**

The primary objective of this project is to create a basis for the diversification of European aquaculture production in high value added finfish species (turbot and halibut) by defining optimal rearing conditions for different populations of turbot and halibut.

The project will investigate the physiological adaptation of different turbot and halibut populations to rearing conditions (temperature and salinity), making it possible to optimise yield (in terms of higher growth and improved disease resistance) for the European flatfish aquaculture industry.

It will focus on the genotype and environment interaction by studying the interacting effect between key environmental factors (temperature, salinity) and different populations of turbot and halibut on growth, growth efficiency, stress resistance, and immune competence. The work plan involves the maintenance of stocks of juvenile turbot and halibut derived from wild caught broodstocks across the entire European geographic range of the species under a range of different temperature and salinity regimes.

The experimental stocks will be held in a large-scale facility for marine sciences (LSF, No ERBFMECT 950013) with individual partners developing and adapting methodologies in their local facilities which will be applied in the common LSF facilities to secure standardisation of the experimental conditions. Concurrent with the physiological experiments, and in order to create a basis for population discrimination of turbot and halibut, the population genetics structure of turbot and halibut throughout their range will be characterised using microsatellite DNA and protein electrophoresis as genetic markers. To ensure industrial exploitation of the results obtained in the project, the current rearing practices, ambient environmental conditions and historical growth performance of stocks will be monitored and documented in a number of selected turbot and halibut farms (SMEs). The consortium will then devise appropriate technical manipulations and production strategies aimed at optimising performance of the turbot and halibut stocks based on the research outputs in the project. These manipulations will be monitored by the consortium so that the enhanced performance of stocks can be compared with traditional (former) farm practices.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.3. New species

FAIR-CT97-3828

Aquaculture of the edible red seaweed Palmaria palmata: development of techniques and economic analysis

OBJECTIVES

The demand in developed countries for naturally derived foods, whether as basic ingredients or as dietary supplements, is increasing substantially as a result of social, environmental and health considerations. Food products obtained directly from the marine environment have a naturally healthy image that can be used positively for marketing purposes. Seaweed has long been exploited within Europe for its food value, although the exploitation has always been restricted to local regions, often with local preferences for particular species. Palmaria palmata, for example, which grows throughout the cool temperate zone in Europe, has long been harvested on a ‘cottage industry’ basis for food consumption in Ireland, although it has also had a traditional, if limited, food use in Scotland, France, Norway and Iceland. A small company, based in Belfast, has established that there is an unfulfilled demand for this product as a direct health food both within Northern Ireland and within the UK as a whole. There is evidence that a substantial additional market niche may be found in the Far East and also, possibly, in Europe. We are also attracted to P. palmata as a target species by the successful development of a natural mutant (‘sea parsley’) that is currently being grown under artificial conditions and marketed in Canada.

Two main problems have held back the development of the resource as a marketable product. First, carefully controlled techniques, as demanded by major sales outlets, have not been established for the preservation, processing and packaging of the product. These techniques are currently being developed commercially within Northern Ireland together with an active marketing strategy. The second difficulty has been to establish both a continuity of supply for the product over the year and reliability of supply of the product from year to year. It is this second problem that we intend to address in the project within a European context. The objectives of the project are to develop and test techniques for cultivating the alga under field conditions (i.e. in the sea in suitable sites) and in land-based tank cultures, and to carry out a series of biological investigations which are directly relevant to its exploitation. The geographical spread of partners is designed to ensure that the potential for the exploitation of P. palmata is studied over a range of climatic and hydrographical conditions.

Two partners will set up some form of raft and suspended rope system, or a benthic frame with rigid upright tubes/poles for settlement of the alga, in the best local site for cultivation. The most suitable material for the rigs and also
the optimal mode of attachment of the plants to the growing surface (e.g. by attaching vegetative thalli, by natural settlement or by inoculating with spores) will be established in these trials. The alternative approach of land-based culture of the alga will also be explored by setting up pilot tanks with a rapid supply of sea water (perhaps enhanced with nutrients by using waste water from fish farms) and plants will be grown in agitation culture under ambient climatic conditions. Close attention will again be given to optimising the growth conditions. The capital and running costs of the schemes will be monitored in all culture studies in order to assess the potential commercial value of the schemes. This information will be fully utilised in conjunction with commercial processing and marketing information to establish the overall market viability of the approach.

Each partner will also undertake a more specific study that will input to the project as a whole. Norway will focus on biochemical studies of the algal thalli and seasonal and geographical variations in its nutritional value, with particular reference to marketing applications. Northern Ireland will undertake molecular and genetic studies of the various morphological forms, and consider the effects and implications of culturing different forms in sites removed from their natural habitat. Ireland will concentrate on physiological studies to optimise the growth rates of different forms, while Spain will investigate the nutrient uptake ability of *P. palmata* and whether its cultivation could be associated with intensive fish farming as a means of removing excess nutrients from waste waters.
5.3.4. Reproduction

FAIR-CT96-1410

Environmental and neuroendocrine control mechanisms in finfish reproduction and their applications in broodstock management

OBJECTIVES

The management of broodstock and the control of spawning are essential requirements for the intensive aquaculture of all finfish species.

A greater understanding of the environmental and neuroendocrine mechanisms underlying seasonal breeding in fish would allow the development of methods for the control of reproduction which could be used to improve the self-sufficiency of seed supplies and hence the profitability of farmed fish production in Europe. Hence, the objectives of this project are as follows:

1. to determine the stimulatory and inhibitory effects of photoperiod and temperature on the brain–pituitary–gonadal axis of the rainbow trout, Atlantic salmon, European sea bass and Atlantic cod;
2. to investigate the neuroendocrinological mechanisms by which the daily and seasonal changes in day length and temperature are perceived by fish and translated into hormonal or neural signals, which act upon the brain–pituitary–gonadal axis, thereby controlling the onset and course of reproductive development;
3. to establish management tools for the environmental control of reproduction, which could then be reliably used by commercial farms to improve the management of their broodstock, and to disseminate this information to the industry via technical advice sheets and workshops.

This project involves the maintenance of stocks of trout, salmon, bass and cod under a range of different photoperiod and temperature regimes. From these experimental fish, samples will be taken for assessments of the changes in activity of the hypothalamic–pituitary–gonadal axis using specific assays for GnRH, GtH, vitellogenin and steroid hormones. Parallel experiments will be conducted on the farms of the SMEs involved in the project. In addition, studies will be made of the role of the pineal gland and melatonin in the transmission of the photoperiodic response to the reproductive axis. This will involve studies of the distribution of melatonin receptors in relation to that of GnRH and other hypothalamic neurones which have been implicated in the control of reproductive function. Techniques to be utilised include autoradiography, immunohistochemistry and the development and use of a series of molecular biology probes.

Environmental control of circulating melatonin levels

Melatonin analysis carried out on Atlantic salmon (in collaboration with partners 1 and 6) clearly showed that melatonin secretion was dependent on tem-
temperature. Further work carried out in collaboration with partner 6 at Matre Research Station on juvenile Atlantic salmon, which are to be maintained to maturity, confirmed that fish, maintained under constant light, display significantly reduced melatonin synthesis during the subjective dark phase compared with fish on a simulated natural photoperiod. Interestingly, the circulating plasma melatonin levels were higher during the subjective dark phase than the basal levels observed during the light phase, suggesting an endogenous rhythm of melatonin production.

A similar experiment was carried out on a high grilsing stock on a commercial salmon farm in Scotland. This used night illumination on selected pens to mimic continuous daylight. Again the lights significantly reduced the night time levels of plasma melatonin when compared with fish under natural photoperiods, and resulted in a grilse rate of 6% compared with 61.5% in the fish under ambient day length. Further trials are to take place over the 1997–98 season using a variety of lights and intensities.

**Melatonin rhythms in cod, sea bass and Atlantic salmon**

A major aim of the project is to apply the technique of melatonin assay developed for use in trout and salmon to measure melatonin levels in cod and sea bass. Melatonin measurements provide a definitive assessment of how photic information is perceived by fish. Preliminary studies have shown a diel pattern in cod with higher levels at night than during the day. Blood samples for melatonin analysis have also been taken from cod maintained in sea pens under continuous illumination at Austevoll Research Station (partner 6). However these, together with cod samples from Bergen, are still awaiting analysis by partner 1; these will be carried out when the assay for cod plasma has been fully validated.

Sea bass samples taken in collaboration with partners 1 and 4 during mid-light and mid-dark phase revealed a diel rhythm of melatonin secretion. This is exhibited by elevated levels during the dark phase when compared with light phase levels.

_In vitro_ investigations by partner 1 into the presence of an endogenous rhythm of melatonin secretion in the Atlantic salmon suggest the presence of an endogenous circadian oscillator in the pineal gland. However, the maintenance of this oscillator after pineal removal may be dependent upon the preceding entraining photoperiod. Further studies will be carried out within the next 12 months.

**Delayed puberty in rainbow trout**

A commercially important finding has been the delay in puberty achieved under long days by trout at the farm of SME partner 2. This means that the egg supplies are being spread throughout the year. The larger eggs produced by first-spawning fish are also an important commercial advance.

**Melatonin and oestrogen receptor expression in rainbow trout**

A major success in the first year of the project has been the development of probes for two of the melatonin receptors known to occur in fish. Investigations have been initiated with the aim of producing further information on the mechanism(s) by which photoperiodic change is transported to the reproductive axis. The development of probes for the melatonin receptors will significantly benefit this work together with ongoing studies to establish whether there are any effects of melatonin on oestrogen receptor (OR) expression.

Preliminary work carried out by partner 3 in conjunction with partner 1 has been looking at the potential effects of melatonin on oestrogen receptor expression in the liver and has revealed no significant light/dark variation in OR expression.
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5.3.4. Reproduction

in rainbow trout. However, an indication that melatonin may affect vitellogenin expression in the liver has prompted further investigations into the effects of pinealectomy and intra-muscular melatonin implantation on rainbow trout.

Partners 3 and 5 have also produced three separate sequences for the melatonin receptor, two of which appear to have a 90% homology with the zebrafish ZEBIA2 receptor sequence (88.2%). The third sequence showed a 79.7% homology to the zebrafish ZEB IB.

Studies into melatonin receptor expression have been undertaken by partners 3 and 5 using Northern blotting and *in situ* hybridisation. Northern blot analysis of brain, pituitary, liver and gonads detected a weak expression in the brain with probes corresponding to different melatonin receptor subtypes (two Mel1A and one Mel1B). *In situ* hybridisation using probes for the Mel1A subtype consistently detected mRNA in the pretectal and thalamic regions, the optic tectum, the cerebellum and the torus semi-circularis. Very low expression, if any, has been found to occur in the neuroendocrine regions controlling the pituitary gonadotrophic functions and in the pituitary itself. These results suggest that the primary targets of melatonin are components of the neurosensorial, in particular visual, systems, confirming data obtained from iodinated melatonin binding studies.

**Ontogenic variation in melatonin binding sites**

A further important finding was the differential levels of melatonin binding found in immature and maturing fish. This may be related to the sites involved in the transduction of photoperiodic information to the reproductive axis.
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5.3.4. Reproduction

FAIR-CT96-1422

Endocrine control as a determinant of larval quality in fish aquaculture

OBJECTIVES

Many aspects of development, differentiation and metabolism in vertebrates are regulated by small biologically active molecules such as thyroid hormones and retinoids. They act via a family of intracellular receptor proteins, which act directly on target genes. Thyroid hormones are passed on to the eggs by broodfish prior to spawning (Kobuke et al., 1987; Brown et al., 1987; Greenblatt et al., 1989, Tagawa et al., 1990) and may provide the necessary physiological regulation for growth, development and osmoregulation in larvae prior to the functional development of their own endocrine glands. There is little doubt that thyroid hormones are essential for fish development, for example, in the metamorphosis of the flounder (Miwa et al., 1988; Yamano et al., 1994). Also, treatment of larvae from several species with thyroid hormones has beneficial effects on development and survival (Brown and Nunez, 1994) and it has recently been shown that thyroid hormone receptors are abundant in sea bream larvae (Llewellyn et al., 1996). However, the investigations into thyroid hormones so far have centred on hormonal measurements or treatments and have not attempted to identify the tissues, which respond to these hormones during development by detecting the presence of specific receptors in those tissues. Growth hormone (GH) is one of the major hormones regulating growth in vertebrates. However, despite evidence that GH cells are present (Power and Canario, 1992; Cambre et al., 1990) and GH is synthesised in recently hatched fish (Funkenstein et al., 1992) and that, in the Japanese flounder, GH expression increases throughout metamorphosis, the functional importance of GH in fish larval development remains to be ascertained.

The prime aim of the project is to establish the importance of thyroid hormones and growth hormone (GH) in the normal and abnormal development of two marine species. These are the Atlantic halibut (Hippoglossus hippoglossus) a cold-water fish with considerable potential as a new aquaculture species and the sea bream (Sparus aurata), a warm-water fish cultivated in the Mediterranean and Portugal, but for which problems of larval culture raise production costs and reduce its economic potential. This will be investigated by:

- the determination of whole body T4, T3 growth hormone and IGF1 profiles during development of the larvae and young fish;
- the determination of the stages at which tissues become responsive to thyroid hormones through the production of thyroid hormone receptors (THRs) and GH by the production of IGF1;

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- the experimental manipulation of the levels of GH, thyroid hormones, and THRs and the determination of the effect of this on development;
- the generation of a model system for abnormal development, based on the actions of retinoic acid, a regulator of vertebrate development already implicated in the production of abnormal larvae in vertebrates, and the use of this model system to determine morphological and molecular aspects of abnormal development.

REFERENCES


FAIR-CT96-1572

An integrated study of interindividual competition and its relationships with feeding physiology and behaviour in cultured predatory fish

OBJECTIVES

This joint research project relies on an integrated investigation of interindividual competition, including cannibalism in young-of-the-year fishes of cultured predatory species, using perch (*Perca fluviatilis*) and sea bass (*Dicentrarchus labrax*) as comparative freshwater and seawater models. The objectives are:

1. to provide the biological bases allowing the heterogeneity of growth and subsequent potential impact of competition under culture conditions to be minimised;
2. to generate models aiming at optimising culture conditions for the early life stages of predatory fish with a comprehensive insight into the interrelationships between feeding physiology, behaviour and production dynamics (mortality-growth);
3. to test the model in commercial culture conditions.

DESCRIPTION OF WORK

The research programme has been defined to study the relationships between some variables identified as having a major potential role in fish competition and cannibalism, and the main production variables usually recorded in aquaculture (e.g. survival, growth, final size heterogeneity and feed efficiency). The recent advances in the intensive aquaculture of the species used as models have allowed the settings of experimental factors to be fixed precisely as population variables (i.e. hatching time, fish stocking density and size heterogeneity within the population), feeding variables (i.e. feeding levels, feeding rhythms, and supplementation of live prey in weaned fish) and environmental variables (i.e. light intensity and photoperiod). The influences of these factors are assessed at two complementary levels (feeding physiology and behaviour) that are used for the understanding of typical production variables. Depending on life stages and species, the investigated physiological variables are the feed intake (calculated in larvae and postlarvae by morphometric relationships between fish body weight and corresponding head weight, and in juveniles by X-ray with ballotini-labelled feed), the gastric evacuation rate and feed assimilation, the proteolytic activities (trypsin, chymotrypsin and pepsin) in the digestive system and the nutrient (N and P) retention and loss. The feeding behaviour is studied by various and complementary approaches such as video monitoring and/or the use of transponders (passive integrated transponder...
tags) detected by immersed antennas placed inside the feeding area and connected to a data-entry station. Investigated variables are the feeding rhythms, the social interactions and the space utilisation by fish in relation to their individual growth rate and feed intake. At the end of the project, some large-scale experiments will be designed to evaluate to what extent the optimal rearing strategy identified from the results obtained during the experimental work will reduce the inter-individual competition in commercial-scale facilities and conditions, and finally improve the net fish production.

STATE OF PROGRESS

In general, all the tasks scheduled in the first year of the project have been carried out as expected. In perch, different independent variables belonging to the boxes population, and feeding and environmental variables have been investigated or are currently under investigation. The effects of initial size heterogeneity on survival, growth, individual feed intake (by morphometric relationships), final size heterogeneity and cannibalism of larvae and postlarvae were investigated and fish samples were preserved for further physiological analyses (enzymatic activities). The same variable was investigated in juvenile perch after some methodological adaptations of the rearing, self-feeding and monitoring equipment and procedures. The effects of food composition (namely the supply of live *Artemia nauplii*) were investigated in perch postlarvae. Several experiments were conducted in larvae, postlarvae and juveniles in order to determine the effects of photoperiod on the physiological, behavioural and production variables previously described. The methodology of labelling newly hatched larvae with alizarine was successfully tested and will be applied in 1998 in order to investigate the effects of hatching time on the dependent variables described above. In sea bass, independent variables investigated in 1997 belong to the population and feeding boxes. The effects of stocking density were studied in larvae and postlarvae, while the effects of feeding level and feed reward were studied in juveniles. Dependent variables and procedures of feed intake measurement were similar to those reported for perch. In juveniles of both species analysis of individual feed intake by X-ray procedure and N-P retention and loss are still ongoing.

ACHIEVEMENTS

Due to the biological cycle of the species and time required for the detailed analysis of samples and results, data processing of most experiments is still ongoing and conclusions cannot be put forward at this stage. Moreover, variables investigated in perch and sea bass in 1997 were not the same, due to facility or technical constraints of the different partners. Hence, this comparison between these two predatory species cannot be done at present. Nevertheless, some interesting results are already available, allowing preliminary conclusions to be advanced in some cases. From the present results, interindividual competition and cannibalism appear highly dependent on the development stage of the fish and on the species. Cannibalism was observed very soon during the ontogeny of perch, while this phenomenon was absent during the first weeks of sea bass larval rearing. In the latter species, mortality was mainly due to feeding adaptation to erogenous diet. At the postlarval stage, cannibalism occurred in both species but this phenomenon affected more deeply the global survival and structure of population in perch than in sea bass. Although growth heterogeneity (coefficient of weight variation) was higher in sea bass than in perch, the proportion of missing fish (as a cannibalism index) was between 1 and 5% in sea bass postlarval experiments while it reached up to 20% in perch of the same stage. In both species, the populational variables already investigated (initial size heterogeneity in perch and stocking density in sea bass) did not induce marked effects on competition and cannibalism. Similar conclusions can be put forward on the basis of results obtained from feeding and environmental variables, except for perch postlarvae reared at different photoperiods. At this stage, the hypothesis of a predominant role of endogenous factors in growth heterogeneity and competition between conspecifics could be proposed, but requires further analysis of data, mainly at the individual and physiological level (ingestion rate, digestive activity and nutrient utilisation, protein synthesis rate, etc.), and experiments. At the juve-
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5.3.4. Reproduction

Nile stage, mortality was very low during the experiments. Aggressivity and cannibalism appeared as a secondary cause of mortality in sea bass, but constituted the major factor of mortality in perch. Competition was mainly related to differences in feed intake and consequent growth heterogeneity among the fish population, although the relationships between individual feed intake and growth rate have not yet been demonstrated. The efficient use of self-feeder by perch and sea bass juveniles constitutes an interesting result (particularly in perch since it had not until now been demonstrated) which allows specific experiments of feeding behaviour to be designed. Most of experiments on juveniles were completed a few weeks ago, or are still ongoing. The analysis of video recordings and electronic monitoring of social interactions and feeding activity are in process. These results, in combination with the individual measurement of feed intake and nutrient utilisation, should provide interesting and integrated information on the relationships between growth and individual competition in fish.

FUTURE ACTIONS

The second year of the project will be devoted to the continuation of the experimental programme, with involvement of the different partners in the following experiments: population variables (effects of hatching time in perch and sea bass, initial size heterogeneity in sea bass, and stocking density in perch and juvenile sea bass), environmental variables (photoperiod in sea bass and perch larvae, and light intensity in perch and sea bass) and feeding variables (feeding level and meal timing in perch). Moreover, physiological and behavioural analyses of experiments carried out in 1997 will be performed in 1998. A preliminary predictive model should be proposed in the second progress report, based on data obtained during the first two years of experiments.
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5.3.4. Reproduction

**FAIR-CT96-1742**

*PTHrP in sea bream development and physiology*

**OBJECTIVES**

Parathyroid hormone-related protein (PTHrP) is apparently a multifunctional protein produced by both normal and malignant tissues and during embryonic and foetal development in mammals and birds. Immunoreactive PTHrP was detected in plasma and tissues of larval and adult sea bream, *Sparus aurata*, and in other fish. These observations suggest that PTHrP has important physiological functions in fish that require investigation. The objectives of the ‘PTHrP in sea bream development and physiology’ project are the following:

1. The identification of the physiological roles of PTHrP in adult and juvenile sea bream. The effect of PTHrP on calcium metabolism (influx and eflux) either in relation to recombinant PTHrP administration or in the absence of biologically active PTHrP, as a result of passive immunisation with anti-PTHrP sera will be studied. The contribution of prolactin (PRL) to calcium metabolism will be assessed by passive immunisation with anti-PRL sera. The effect of PTHrP on growth will be studied by hypophysectomy and replacement therapy. An *in vitro* culture system will be used to study the effects of calcium regulating factors in *Sparus* cells and tissue.

2. The determination of the functions of PTHrP in normal and dystrophic embryonic and larval development. The effects of PTHrP on *Sparus* embryonic and larval development will be studied by culturing eggs and larvae at defined stages in the presence or absence of PTHrP, or by applying PTHrP to specific tissues. The effect of PTHrP will be monitored using macroscopic, histological and histochemical methods. Particular attention will be paid to critical stages of metamorphosis such as mouth opening, functionality of heart, gut differentiation and swim bladder, nervous system and muscle formation. The interaction of environmental factors, such as salinity, calcium and temperature fluctuations, with PTHrP expression and occurrence of axial dysplasias will also be studied.

3. Cloning the gene for PTHrP receptor(s) in sea bream. cDNA libraries will be prepared from *Sparus* tissues and used to clone the gene for the PTH/PTHrP receptor; its tissue distribution and level of expression in different physiological/developmental conditions will be determined.

**INTRODUCTION**

Parathyroid hormone-related protein was identified as a factor in fish (*Sparus aurata*) pituitary and plasma using antisera to human PTHrP (Danks, Devlin et al., 1993). Since then, we have shown that immunological PTHrP is present in tissues and plasma of elasmobranch (Ingleton, Hazon et al., 1995), in the
saccus vasculosus of *Sparus* (Devlin, Danks et al., 1996) and in the urophysis and corpuscles of Stannius of the euryhaline teleost *Plachthys flesus*. These observations suggest that PTHrP has important physiological functions in fish that require investigation. The cross-reactivity between antibodies to human PTHrP and fish tissues indicates that there has been evolutionary conservation of the molecular structure and composition so predicting that the functions of PTHrP are fundamental and vital. Thus, the functions of PTHrP in mammals are likely to have originated from those established in fish and hence form the basis for areas of investigation in fish species. Furthermore, the vital importance of PTHrP is reinforced by studies in mice showing that offspring in which the gene has been deleted fail to survive the perinatal period (Karaplis, Luz et al., 1994).

Because fish do not have a parathyroid gland, it is possible that PTHrP acts as a hypercalcaemic agent and its location in the kidney tubules, corpuscles of Stannius, the saccus vasculosus of some teleosts and the rectal gland of elasmobranch suggests that it may be involved in the control of ion balance. However, unlike mammals and birds, fish appear to have high circulating concentrations of PTHrP, which may be derived from the pituitary (Danks, Devlin et al., 1993), suggesting that, in this group of vertebrates, it acts as a classical hormone in adults in addition to local tissue interactions. It is more likely that PTHrP functions as a paracrine or autocrine factor in the embryonic and larval development of fish, as it does in chickens and mammals.

PTHrP is a candidate factor involved in both the normal and abnormal development of sea bream embryos and larvae. The production of PTHrP may depend both upon intrinsic and environmental factors at the early stages of development of sea bream and it is important to determine both PTHrP gene expression during development and to investigate the environmental factors which may be involved in affecting both this expression and the induction of axial malformations. It seems likely that PTHrP is a factor involved in the embryonic development of sea bream tissues and particularly affects the integrated differentiation of the vertebral column, spinal cord and segmental muscles. PTHrP also has potential involvement in the development and functioning of the swim bladder, which also fails to grow properly in dystrophic juveniles.

In order to determine the actions of PTHrP in *Sparus*, it is necessary to demonstrate which tissues are susceptible to PTHrP action through specific receptors. The gene for the common PTH/PTHrP receptor in mammals, the only receptor so far demonstrated for PTHrP, is highly conserved between tissues and species examined and-prescribes a G-protein-linked seventrans membrane domain construct (Kong, Schipani et al., 1994). Such receptor forms appear to be conserved across vertebrate groups.

The objectives of this project are the identification of the physiological roles of PTHrP in adult and juvenile sea bream, the determination of the functions of PTHrP in normal and dystrophic embryonic and larval development and the cloning of the gene for the PTHrP receptor(s) in sea bream.

RESULTS

The results obtained so far indicate that *Sparus* accumulates calcium for growth and development and that the gills play a significant role in the calcium balance. In particular, very young fish seem to take up calcium from the water at a very high rate. No effect of hPTHrP injections on any form of calcium uptake from the water or on plasma calcium levels has yet been found.

For other aspects of the physiology of PTHrP, an antiserum which reacts specifically with PTHrP was required and this has been developed in rabbits using a short pentapeptide region of the molecule which is common to tetrapod PTHrP already analysed. The specificity of the antiserum has been checked by immunohistochemistry of *Sparus* tissues.
Examination of tissues from normal and dystrophic juvenile *Sparus* has shown that the distribution and abundance of immunoreactive PTHrP (irPTHrP) and its gene expression differ in the two conditions. The protein is present in the brain and spinal cord, pituitary, skin, muscle, gills, kidney, motor neurones and dorsal root ganglia and remnant notochord cells. However, the dystrophic spinal columns in addition to these sites of irPTHrP also retain chondrocytes in the areas of cartilage overgrowth in the spinal column.

Primary cell cultures from sea bream bone tissues can now be easily prepared. These cells are therefore a first-hand material that will enable us to look for specific gene expression and regulation. A clear description of the chronology of development of the diverse bone structures, head, vertebral column, and fins has been generated in sea bream. PRC probes for myosin, alpha-actin and gamma-actin are now available and can be used to study sea bream development and the effect of PTHrP on development. cDNA libraries including a kidney library developed in this project are now available to isolate PTHrP and its receptor.

**DISCUSSION**

Since the project is still at an early stage, it is only possible to speculate on the roles of PTHrP. So far we have not been able to show the effects of hPTHrP injections on any form of calcium uptake from the water or on plasma calcium levels. In the coming year, other doses and administration regimes will be evaluated once more for their effect on branchial calcium handling, and also conditions will be set in which calcium uptake is stimulated (enhanced growth, salinity variations).

The effect of PTHrP on bone cell lines can now be studied before *in vivo* experiments are carried out in order to conserve costly hormones.

After some preliminary experiments, the technique of hypophysectomy will be developed for the sea bream and hormone replacement therapy experiments will be carried out using several different hormones.

The effects of PTHrP on early development will be studied and the distribution of muscle proteins will be studied using oligonucleotide probes.

Finally, it is expected that the sea bream PTHrP receptor will be isolated and characterised.

**ACKNOWLEDGEMENTS**

The study has been carried out with financial support from the European Commission, through its agriculture and fisheries (FAIR) specific RTD programme, CT96-1742, ‘PTHrP in sea bream development and physiology’. It does not necessarily reflect the Commission’s views and in no way anticipates its future policy in this area.

**REFERENCES**

5.3. BIOLOGY OF SPECIES FOR OPTIMISATION
OF AQUACULTURE

5.3.4. Reproduction


Breeding improvement of Crassostrea gigas by nutritional and gametogenesis control

OBJECTIVES

Nutrition is one of the major aspects which can be at the origin of reproduction difficulties in oyster hatcheries. In commercial hatcheries, broodstock conditioning requires large quantities of phytoplankton and the choice of the algal species is generally dictated by its suitability for mass production at low cost under the environmental conditions of the hatchery rather than by its optimal nutritional value for the oyster. This empirical approach is at the origin of deficiencies in the algal diet for the broodstock which may affect various aspects of reproduction, including spawning success and egg quality, and the growth rate, sensitivity to stress and pathogens of the larvae. This project aims to improve broodstock management in oyster hatcheries through a better understanding of the relationship between broodstock nutrition and quality of early life stages, with the following specific objectives:

1. to document problems in current hatchery practice by comparison of nutritional aspects of reproduction under natural and artificial conditioning;
2. to identify critical nutrients for broodstock nutrition;
3. to define artificial diets to supplement these nutrients to live algae, taking into account the initial nutrient storage of broodstock;
4. to improve the cost-efficiency of broodstock conditioning.

Nutritional deficiencies in algal diets, importance of nutrient storage and oyster requirements will be assessed through the characterisation of histological, histochemical and biochemical events during the reproductive cycle under natural and hatchery conditions. Four research groups with complementary experience in artificial diets, bivalve nutrition and broodstock conditioning, and bivalve reproduction will work on the problem of oyster nutrition in collaboration with three European hatcheries and one company specialised in larviculture diets. The hatcheries will contribute to validating the experimental results under their specific culture conditions.

RESULTS

The first-year activities, i.e. the sampling campaign in nature and two broodstock conditionings, have been completed. The histological and histochemical observations, biochemical analysis and the glycogen metabolism were evaluated and a comparison between the artificial broodstock conditioning and the
natural conditioning was made. The effects of both conditions on the production parameters and on the biochemical composition of the eggs and D-larvae were also evaluated. Formulation of diets, and verification of ingestion and assimilation were carried out and a supplementation feeding strategy for the next broodstock conditioning has been proposed. The development of standardised stress and challenge tests is still in progress and these will be tested on the output of the next spring broodstock experiment.
5.3.4. Reproduction

**FAIR-CT96-1941**

*Early control of growth for fish production with special reference to muscle development, gene expression and temperature*

**OBJECTIVES**

The main objective is to assess the effects of environmental temperature on muscle development and growth efficiency during early life. Emphasis will be placed on muscle cellularity, differentiation, gene expression, and growth during the embryonic and larval stages in two main farmed species of fish: rainbow trout and sea bass.

The application of such temperature effects will be investigated through the consequences of alteration in muscle phenotypes by early temperature treatment on growth efficiency during postlarval growth in rainbow trout and sea bass. A complete description of the temperature effects during early development will be given to provide the possibility of application for aquaculture.

**CURRENT PROGRESS AND RESULTS**

The survival, growth and developmental rates of rainbow trout and sea bass embryos reared during early development at lower temperatures than those usually used have been monitored. In rainbow trout differential mortality was observed at low temperature (4 °C) and high temperature (12 °C). This seemed to be dependent on the strains and a shift towards higher temperature in the range of viable temperatures for early development could be detected in some rainbow trout strains. In sea bass preliminary experiments were realised on a small scale in order to define the low-temperature protocol (temperature, timing) to be applied in a large-scale experiment. The large-scale sea bass experiment is in progress and similar survival rates were observed at the three temperatures tested. These experiments provide original data on temperature and early development in the two farmed species.

A comprehensive analysis of some developmental stages was made in rainbow trout and sea bass as the timing of these stages was greatly affected by temperature. Description and timing of muscle development was also analysed in rainbow trout (see the original results presented below). Serial sampling at specific developmental stages was made in rainbow trout and will be made in sea bass depending on temperature. In rainbow trout developmental rate was lowered at low temperature. Differences in body weight and length growth rates were also observed depending on temperature and consequently the morphology of larvae was affected by temperature.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.4. Reproduction

Muscle development will be followed by changes in cellularity (number and size of myotubes and fibres) and in expression of muscle-specific genes (myogenic factors and contractile proteins). The muscle cellularity analysis is still in progress in rainbow trout. The lowering of developmental rate observed at low temperature for some strains required further analysis of the number and size of fibres in order to draw conclusions on the positive effect of low temperature. In situ hybridisation methods were adapted to these early stages.

Specific rainbow trout cRNA probes from available cDNA of growth factors, myogenic factors and contractile proteins were tested successfully. In sea bass specific cDNA probes for growth factors, myogenic factors and contractile proteins were raised and started to be tested. Thus new tools, which have only been available until now in zebrafish, have been developed in two farmed species, i.e. rainbow trout and sea bass, for developmental biology studies.

Growth performances, muscle growth and muscle characteristics of fish previously exposed to different temperature regimes during early development will be analysed from the first feeding stage up to the commercial stage (300 g) in rainbow trout. This experiment is in progress in two strains and under two different environmental conditions in rainbow trout.

ABSTRACT

Embryonic muscle development was studied in rainbow trout (Oncorhynchus mykiss) at low and high temperature using scanning electron microscopy (SEM) and immunohistology. Somite development was described starting at stage 16 and up to the hatching stage. Somite growth in height was more important than in width and depth. Myotubes without any structure and then myotubes with cross-striated contractile elements organised in myofibrils were successively observed in the somite. Immunohistology analysis demonstrated the appearance of an embryonic fast myosin in the deep part of the somite at stage 20 (Vernier, 1969). The area of expression of myosin then expanded in the somite and covered the whole somite at hatching (stage 30). Slow myosin was only expressed at the eyed stage (stage 24) in few superficial cells forming a U-shape at the horizontal septum and then expanded dorsally and ventrally. Specific stages for muscle development were thus identified in rainbow trout.

INTRODUCTION

The early development of rainbow trout is well characterised anatomically and the main stages have been fully described (Henneguy, 1988; Pasteels, 1936; Vernier, 1969; Ballard, 1973). Very little attention has been paid to muscle development apart from the number of somites used to identify the embryonic developmental stage. Analysis of the characteristics of cells within the somite in trout demonstrates an anteroposterior gradient in the development of skeletal muscle along the vertebral axis starting with the appearance of deep ’white cells’ and superficial ’red cells’ (Nag and Nursall, 1972) differentiating first in myotubes and then in fast and slow fibres. Differentiation from early myotubes to muscle fibres in the deep part of the somite has also been characterised in herring embryos.

In the zebrafish (Danio rerio), which has been used as a model for developmental studies, new information about muscle development and myogenesis inside the somite has been obtained. Myogenesis originates in the deep part of the somite near the notochord where two distinct populations of muscle precursors have recently been identified (Devoto et al., 1996). These precursor cells express different growth factors and myogenic factors as they became myoblasts. After a proliferating phase, myoblast fusion leads to the formation of myotubes, differentiating later into muscle fibres.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.4. Reproduction

The aim of this study was to describe morphological and functional aspects of muscle development during the embryonic stages of rainbow trout. Myogenesis was analysed through the expression of myosin inside the somite.

MATERIALS AND METHODS

Fertilisation was realised on a pool of ovules from two females and milt from nine males. Eggs were incubated at two constant temperatures (12 °C and 4 °C) and constant oxygen concentration (98 % saturation) up to end-of-yolk-sac resorption.

Samples were collected every 50 degree.days at the two temperatures from fertilisation up to the eyed stages then at the same developmental stages as those according to Vernier (1969). Eggs were dissected and embryos removed from the chorion. Direct observation was made by microscopy. Other embryos were treated using SEM or histoinmunology.

**Scanning electron microscopy:** Embryos were fixed in Karnovsky fixative (paraformaldehyde 4 %, glutaraldehyde 5 % sodium cacodylate 0.08 M) during 6 to 20 hours at 4 °C then in osmium tetroxide 0.1 %. Samples were dehydrated by successive immersions in ethanol 30 % to 100 %. Samples were dried up to the critical point method and an or-palladium mixture was pulverised on the samples. They were observed using a scanning electron microscope (JEOL 8300, University of Rennes MEB centre) directly and after removal of the skin and fractures of the embryos.

**Immunohistology:** Embryos were fixed in ethanol 70 % (in glycine buffer 0.05 M, pH = 2.0) then dehydrated (ethanol 95 %, butanol) and paraffin embedded. Thin transverse sections (5 µm) of the embryos were made and put on TESPA-treated glass for immunology analysis. Sections were disembedded, dehydrated (acetone, ethanol 100 % to 70 %) and rinsed with distilled water and PBS (0.01 M, pH = 7.4). Sections were then treated with BSA 0.2 % and saponine 0.2 % in PBS. The sections were treated with the first antibody (20 µl/section) during one hour and rinsed with PBS, and then with the second antibody coupled with FITC dye (20 µl/section anti-mouse TEBU M 30001, anti-rabbits TEBU L 42001) during one hour. The sections were rinsed with PBS, mounted in Mouwiol solution and observed with a light fluorescent microscope.

RESULTS AND DISCUSSION

The different stages observed by direct microscopy were checked by SEM analysis (Table 1). The rate of somitogenesis was 0.8 somites/degree.day and it was similar at the two temperature studied. The shape of the somite changed from a pillow shape in the anterior part of the embryo at stage 16 up to a classical chevron shape observed all along the vertebral axis at the eyed stage. During the embryonic period, there was a ninefold increase in the height of the somite (from 70 µm at stage 16 up to 600 µm at hatching), only a threefold increase in depth (from 30 to 90 µm) and few changes in width (lateral view from 30 to 60 µm).

On transverse fractures of embryos, thin elongated structures assimilated to myotubes were observed in the somite at stage 24. These structures seemed to be present in the deep part of the somite only, close to the notochord. At stage 26, cross-striations were observed in these structures. At hatching (stage 30), fibres composed of myofibrils (1 µm) were observed. The cross-stratiation was clearly distinguishable and sarcomere length was assessed to 1 µm.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.4. Reproduction

Myosin expression was first observed at stage 20 using a fast MHC antibody (S48E6) but not at the early stages (16, 18). The expression was limited to the very deep part of the somite near the notochord at stages 20 and 22 (Table 2). Then at stages 24 and 26, the area of myosin expression expanded laterally with a gradient of intensity from the deep part of the somite to the periphery in agreement with the gradient in muscle differentiation described in zebrafish (Waterman, 1969; Devoto et al., 1996). Myosin expression was observed in the whole somite at hatching (stage 30). The same results, although more specific, were observed with a fast MHC antibody (S410H9) except that no expression was observed until stage 22.

No myosin expression was observed until stage 24 using a slow MHC antibody (BA-D5). At stage 24 (eyed stage), slow myosin expression was localised at the periphery of the somite in elongated monolayer cells forming a U-shape at the horizontal septum. The expression of slow myosin expanded dorsally and ventrally at stage 30 (hatching). Such differ-

### Table 1: Description of sampling stages (degree.d — degree.days)

<table>
<thead>
<tr>
<th>Stage number (Vernier 1979)</th>
<th>Stage name</th>
<th>Degree.d at 12 °C</th>
<th>Muscle</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–11</td>
<td>Blastula</td>
<td>48</td>
<td>20 to 30 somites</td>
<td>Pillow shape</td>
</tr>
<tr>
<td>16–18</td>
<td></td>
<td>96</td>
<td>20 to 30 somites</td>
<td>Pillow shape</td>
</tr>
<tr>
<td>20–22</td>
<td></td>
<td>144</td>
<td>55 to 65 somites</td>
<td>Heart beating</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oval shape</td>
<td>Two to three gill vents</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Muscle contraction when stimulated</td>
<td>Pectoral fins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caudal bud detached</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pronephros</td>
</tr>
<tr>
<td>24</td>
<td>Eyed stage</td>
<td>196</td>
<td>24</td>
<td>Optical vesicle pigmented</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2/3</td>
<td>Four gill vents</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>Opening of mouth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>Opening of kidney</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>252</td>
<td>26</td>
<td>Anal and dorsal fins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>Lower maxillary</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>Olfactory cavity</td>
</tr>
<tr>
<td>30</td>
<td>Hatching</td>
<td>336</td>
<td>30</td>
<td>Pelvic fins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3/4</td>
<td>Olfactory cavity doubled with ciliated cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3/4</td>
<td>Gill arch</td>
</tr>
</tbody>
</table>

### Table 2: Fluorescent immunolabelling of fast and slow myosin in the somite during embryonic development (stages according to Vernier, 1969)

<table>
<thead>
<tr>
<th>Stages</th>
<th>Fast myosin (S48E6)</th>
<th>Fast myosin (S410H9)</th>
<th>Slow myosin (BA-D5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 16</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Stage 18</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Stage 20</td>
<td>Deep (1/3)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Stage 22</td>
<td>Deep (1/3)</td>
<td>Deep (1/3)</td>
<td>None</td>
</tr>
<tr>
<td>Stage 24</td>
<td>Deep (1/3)</td>
<td>deep (1/3)</td>
<td>Monolayer</td>
</tr>
<tr>
<td>Stage 26</td>
<td>1/3</td>
<td>1/3</td>
<td>Monolayer</td>
</tr>
<tr>
<td>Stage 30</td>
<td>All somite</td>
<td>All somite</td>
<td>Monolayer</td>
</tr>
</tbody>
</table>
ential expression of myosin isoforms has never been described in salmonids but it was detected by early TEM analysis
(Nag and Nursall, 1972). This pattern is different to that observed in zebrafish where slow myosin expression starts in
the deep part of the somite (Devoto et al., 1996).

Desmin expression was observed very early in the deep part of the somite at stages 20, 22 and 24 with a similar pattern
to that of the fast myosin antibody (S48E6), although the intensity of labelling was greater. No differences were observed
in terms of myosin or desmin expression between low and high temperatures at stages 24, 26 and 30.

CONCLUSIONS

Specific stages for muscle differentiation were identified. These results also demonstrated the role of notochords in the
white muscle differentiation in rainbow trout. A sequential expression of fast and slow myosin with a late expression of
slow myosin in the superficial cells of the somites with a different pattern to that of other models such as zebrafish was
observed.

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FAIR-CT97-3382

Study of antioxidant systems in cultured marine fish in relation to growth, quality and disease resistance during early developmental stages

OBJECTIVES

The development of rearing methods for marine fish larvae is still one of the major constraints for increasing the aquaculture production of marine fish. Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation, is acknowledged as being highly deleterious resulting in damage to cellular biomembranes, which contain large amounts of PUFA. Information in relation to in vivo lipid peroxidation and antioxidant defences either in wild or cultured fish species is quite limited.

The objectives of the project are:

1. to demonstrate the significance of oxidative stress, and the role of the antioxidant systems in marine fish under highly controlled experimental conditions, primarily in weaned fish;
2. to characterise the antioxidant system in marine fish during early development in order to understand and enhance the growth and quality of early life stages by avoiding oxidation problems that may cause pathologies and disease.

To achieve the objectives, the project will comprehensively investigate all three aspects involved in the control of lipid oxidation in marine fish, namely endogenous antioxidant systems, dietary inputs of highly unsaturated fatty acids (HUFAs) and antioxidants, and lipid oxidation products.

Therefore, the project will determine, if possible, the PUFA, antioxidant and lipid oxidation status in fish from early stages to weaned fish in three marine species: gilthead sea bream, turbot and halibut.

The proposed work will increase knowledge of the biology of farmed European marine fish species with a view to improving larval quality and optimising their culture. If the project is able to demonstrate a beneficial effect of antioxidants, it could be a major step forward in the development of rearing methods for marine fish larvae.
5.3. BIOLOGY OF SPECIES FOR OPTIMISATION OF AQUACULTURE

5.3.4. Reproduction

**FAIR-CT97-3785**

**Molecular and physiological basis for the optimisation of GnRH-induced spawning techniques in farmed fish, in particular the sea bass (Dicentrarchus labrax) and the sea bream (Sparus aurata)**

**OBJECTIVES**

Full control of reproduction is essential for all finfish species under intensive culture. This control will enable fish farms to synchronise and improve the quality of spawnings and, therefore, better programme their seed supplies according to the market demands.

The project aims to understand the neuroendocrine and the physiological mechanisms involved in the reproduction of sea bass and sea bream. The objectives are:

1. to investigate at the pre- and post-synaptic levels the neuroendocrine mechanisms by which the best combination between GnRH treatment and gonadotroph sensitivity is achieved to develop reliable technology to induce spawning in sea bass and ensure an optimal efficiency of improved spawning induction and synchronisation therapies in sea bass and sea bream;

2. to purify and develop specific assays of GtH(s) in sea bass and design new GnRH analogs to test their biological activities in terms of GtH secretion, expression and quality of spawning;

3. to establish management tools for selected GnRHa and delivery systems which could then be reliably used by commercial farms to induce spawning and improve synchronisation and quality of the broodstock spawnings.

The techniques to be used in this project include purification of sea bass GtH(s), setting up specific assays for different fish hormones’ cloning and expression of recombinant hormones (GnRH(s), GtH(s) and GAP(s), cloning and expression of GnRH receptors). It also includes studies on brain neuroanatomy, immunohistochemistry and *in situ* hybridisation at the brain and pituitary levels of sea bass and sea bream and experimental studies including the effect of different hormonal treatments and environmental changes on the GnRH–GtH–gonadal system. At the same time a wide range of controlled release GnRHa devices will be prepared and tested in broodstock sea breams and sea bass. Their effects on hormonal induction of final oocyte maturation and spawning and egg and larval quality studies will be assessed.
**FAIR-CT95-0174**

**Towards improved carbohydrate utilisation by finfish: physiological, metabolic, molecular and genetic limitations of poikilothermy**

**OBJECTIVES**

The overall objectives of the project were to understand the control mechanisms of the metabolic utilisation of dietary carbohydrates as affected by water temperature in three commercially important finfish species: rainbow trout, common carp and gilthead sea bream.

Firstly, the studies were aimed at the quantitative evaluation of the beneficial effects of different technological treatments to improve starch digestibility. Secondly, the project’s aims were to understand the metabolic specificities between species indicating disorders of glucose homeostasis: prolonged post-prandial hyperglycaemia, insulin secretion, receptivity, and glucose phosphorylating capacity. The specific objectives were thus to: (i) assess the nutritional and endocrinological status or potential of fish fed two different sources and levels of carbohydrates using nutritional, biochemical and metabolic criteria; and (ii) to evaluate the dietary or temperature influences on the activities of glucose phosphorylating enzymes and their induction at a molecular level.

The present interdisciplinary project was initiated to undertake growth studies with the three species fed two levels of two kinds of starch-rich ingredients (wheat, a cereal, and peas, a pulse) and to analyse their effects on nutrient/energy utilisation, phosphorylating capacity, postprandial glycaemia, insulin secretion and tissue receptivity and levels of glucose transporters. A further objective was to carry out the molecular cloning of the hexokinase (HK) gene, whose activity is considered as the first limiting step in glucose phosphorylation. Selected data from the large amount of data generated so far (end of second year) are briefly summarised below.

**SYNOPSIS OF RESULTS AND DISCUSSION**

1. *Extrusion technology to improve starch gelatinisation and digestibility of starch*

Different conditions of the extrusion and flaking process were applied to whole wheat grains and the digestibility of the obtained wheat products was measured in trout at 8 °C and 18 °C and in carp at 18 °C and 25 °C. The results show that when properly ground (< 500 µ), the starch digestibility of whole wheat without any extrusion treatment is high in carp. However, for rainbow trout, a definite extrusion treatment is required to have the gelatinisation ratio above 98 % in order to obtain starch digestibility of 80 % (Figure 1).
2. Utilisation of dietary carbohydrates by rainbow trout and common carp

Four experimental diets having a common basis were formulated to contain one of two levels (20 and 35 %) of either extruded peas or extruded wheat. Apparent digestibility coefficients (ADCs) of the experimental diets were measured at both temperatures. Two separate growth studies under experimental fish farm conditions were performed with juvenile rainbow trout and common carp, each reared at two different temperatures, in order to measure the efficiency of experimental diets containing the two different amounts of extruded wheat or peas. At the end of each trial, besides the determination of all nutritional parameters, tissues were sampled to study plasma glucose and insulin levels, hepatic hexokinase activity and expression of hexokinase mRNA and insulin receptors.

Postprandial changes in plasma glucose and insulin

In fish fed a starch-free diet, the postprandial plasma glucose levels were low (below 1 g/l) and relatively stable over the day. At 18 °C, in trout fed starch-rich diets, the peak values were observed between six and eight hours and slightly later in fish grown at 8 °C. In trout grown at 18 °C, the postprandial rise in plasma glucose levels was higher in those fed high levels of wheat starch than in all other groups. With regard to plasma insulin levels, no specific effects of the dietary treatments were found. Even in those fed a starch-free diet, the postprandial insulin pulse was high and this was not connected to any similar rise in plasma glucose values. This indicates that the increase in circulating levels of insulin is probably due more to an effect of dietary amino acid supply and utilisation by peripheral tissues than being directly related to the supply of dietary glucose.

Hepatic glucose phosphorylating capacity in rainbow trout and common carp

Selected data on the hepatic HK and glucokinase (GK) activities are given in Figure 3. In rainbow trout grown at 18°C, liver HK activities were low but slightly affected by dietary starch levels. Activities of GK were very high and also affected by dietary starch availability. In trout which were fed the carbohydrate-free diets, the glucose phosphorylating capacity seemed very low. In common carp grown at 25 °C, activities of GK were lower than in trout and the effects of dietary carbohydrates were less pronounced.
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Glucose oxidation and lipogenesis

Fish which were fed the experimental diet which led to the best growth performance and the highest lipid gain were used to evaluate the involvement of glucose in oxidative catabolism and in lipid synthesis. Postprandial changes in metabolic utilisation of glucose were studied by sampling fish 6, 12 and 18 hours after administration of a U-14C-glucose solution. Data revealed that glucose is actually used as an energy supplier especially at high temperature and that lipid synthesis from glucose is low.
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Tissue receptivity to insulin and glucose transport

Plasma insulin levels increased after meals in all experimental groups and species. The number of receptors appeared to be different between species possibly in relation to the natural dietary preferences of the three species. Initial studies show that in the rainbow trout dietary carbohydrate level (20 or 40 %) or source (wheat or pea) appeared to have no significant effect on postprandial insulin levels. In the muscle of trout, the number of insulin or IGF-I receptors did not show differences between groups (Figure 4); in all cases, IGF-I binding was higher than insulin binding, and the Kd was lower for IGF-I receptors. In carp, the number of insulin receptors and specific binding were higher than in trout and were also positively correlated with the levels of carbohydrate in their diets. Insulin and IGF-I receptors showed tyrosine kinase activity, which was stimulated by the respective peptide and influenced by adaptation to dietary treatment.

Analysis of glucose transporters

The presence of a putative glucose transporter in fish that is homologous to the mammalian insulin-regulated glucose transporter GLUT4 was investigated using a molecular biological approach. Results show that the homology between mammalian and fish GLUT4 sequences is too low to enable the detection of fish GLUT4 mRNA with a mammalian GLUT4 cDNA probe by Northern blot analysis. Since GLUT4 cDNAs from non-mammalian species are not available, studies are under way to clone the trout GLUT4 cDNA by RT-PCR with degenerate oligonucleotide primers (DOPs) based on conserved regions of GLUT4.

3. Molecular cloning of the hexokinase (HK) and glucokinase (GK) genes in teleosts

By choosing adequate degenerate primers from a region corresponding to the glucose and ATP-binding sites in several species, and applying the DOP-PCR (degenerated oligonucleotide primed polymerase chain reaction) technique, DNA probes for GK from all the three species (trout, carp and sea bream) and for HK from the last two were obtained. RNA

![Graph](image.png)

Figure 4: Number of insulin-binding receptors from the skeletal muscle of trout and common carp
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hybridisation through Northern blot and specific RT-PCR for GKS from fish fed diets with and without carbohydrates were made. The expression of the rainbow trout GK gene as affected by the nutritional status was studied.

20 µg of total RNA from rainbow trout livers was submitted to electrophoresis on 1 % agarose gel, transferred onto nylon membrane and hybridised with the probe. The membrane was hybridised with 16S common carp rRNA labelled and served as the internal control.

As illustrated in Figure 5, the expression of the GK gene in rainbow trout livers differed significantly according to the dietary level of digestible starch in diets. There is a weak expression of the GK gene in the livers of rainbow trout fed without carbohydrates (detected in PCR but not in Northern blot six hours after feeding) which is contrary to the livers of rainbow trout fed with carbohydrates. The GK mRNA expression was also found to be related to the level of hepatic GK activity measured in rainbow trout. These results strongly suggest that the GK gene expression in liver depends upon the dietary carbohydrate level in at least some teleosts.

![Figure 5: RNA hybridisation with the rainbow trout GK probe](image)

**Diet with carbohydrates**  **Diet without carbohydrates**

GK mRNA

16S RNA

Figure 5: RNA hybridisation with the rainbow trout GK probe
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5.3.5. Fish nutrition

**FAIR-CT96-1329**

*Effect of processing technology on the quality of aquaculture feeds*

**OBJECTIVES**

The main objective of the project is to verify the reliability of several innovative chemical methods (aspartic acid racemisation, proportion of SH to S-S bonds and near infrared reflectance (NIR) techniques) to predict the protein digestibility of protein fed to farmed fish. The prediction of the true nutritive value of the protein ingredients used in aquafeed preparation has a central role in allowing correct use of food resources, reducing wastes and thereby contributing to establishing a real sustainable aquaculture, both from an economic and ecological point of view. The kinetics of aspartic acid racemisation and S-S bond formation will be studied to elucidate the role of different processing parameters (pH and moisture content of the raw material, oxygen pressure, temperature and duration of the thermal treatment) on the protein quality of fishmeal, soyameal and extruded diets, aiming at defining indicative guidelines for the production of feed ingredients and fish feeds characterised by higher protein quality. The availability of reliable and rapid predictive chemical methods to evaluate the quality of the protein ingredients of fish feeds would, in fact, allow the European fish feed manufacturing industry to produce a dietary protein of high quality which is expected to be well utilised by fish, to allow reduction in the protein content of the feed and to reduce the environmental impact of fish farming.

**METHODOLOGY**

The abovementioned objectives will be reached through the following steps.

1. Method development for chemical analysis of D-aspartic acid, SH groups and S-S bonds and for the application of NIR techniques to the feed and feed ingredients such as fishmeal and soyameal.
2. Elucidation of the basic physical/chemical reactions involved in modifying the quality of the protein through the production on a laboratory scale of feed materials (using whole herring *Clupea harengus* as raw material) treated under defined physical and chemical conditions: the kinetics of aspartic acid racemisation and S-S bond formation will be studied in relation to the major processing variables such as time and temperature of treatment, oxygen pressure, and pH and moisture content of the raw material.
3. Production on a pilot plant scale of samples of fishmeal and extruded fish feed treated under different processing conditions and evaluation of the chemical parameters under study (i.e. D-aspartic acid content and SH to...
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S-S ratio). Two different samples of fishmeal (high and low temperature) will be produced and each one will be subjected to three different extrusion conditions (gentle, medium and tough) to produce the experimental fish feeds.

4. Digestion studies (both in vitro using fish enzymes and in vivo with rats, chicks, mink and fish) to determine the protein digestibility in the various species and to evaluate the reliability of the abovementioned chemical parameters to predict the digestibility of the protein.

5. Production of extruded feeds in commercial conditions (extrusion conditions based on the results of the pilot-plant-scale study) and quantification of the chemical parameters under study (i.e. D-aspartic acid content and SH to S-S ratio) to evaluate possible differences between the pilot-plant-produced feeds, therefore allowing a correct evaluation of the effects of the treatments occurring in a real commercial production plant.

6. In vivo utilisation study of the experimental feeds on a tank scale (pilot-plant-produced feeds) and on a farm scale (commercial-plant-produced feeds) to evaluate the effects on the growth performances and capacity for protein synthesis in the muscle of farmed fish using both north European (such as Atlantic salmon, Salmo salar, and rainbow trout, Oncorhynchus mykiss, this latter under northern Italian farming conditions) and Mediterranean (European sea bass, Dicentrarchus labrax) species. During the farm-scale trials, possible effects of using different feeds on the water quality parameters of the effluents from the fish farms will also be recorded.

7. Determination of regression relationships between chemical determinations of D-aspartic acid, SH and S-S content in fishmeals and fish feeds and ileal digestibility in rats or chicks, and digestibility determined in mink, Atlantic salmon, rainbow trout and European sea bass. Determination of regression relationships between chemical determinations of D-aspartic acid, SH and S-S in fishmeals and fish feeds and growth of Atlantic salmon, rainbow trout and European sea bass. Determination of regression relationships between ileal digestibility of protein in rats, chicks and faecal digestibility in mink and fish. Determine regression relationships between the various estimates of protein digestibility and growth of Atlantic salmon, rainbow trout and European sea bass. To fully validate the proposed chemical methods, these methods will also be applied to commercial samples of fishmeal on which in vivo digestibility has been determined.

BENEFITS

The major benefit expected from the project for the European aquafeed manufacturing industry will be the availability of rapid and cheap chemical, physical or biological methods for the evaluation of protein quality in aquaculture feeds. As a consequence, it will be possible to use feed ingredients with a better, controlled, protein value, therefore allowing a reduction in the protein content in the feed and a reduction in the percentage of non-utilised nitrogen excreted by farmed fish.

PRODUCTS

The main product of the project is expected to be, as cited above, the availability of new chemical, physical or biological quality parameters for the rapid evaluation of the protein quality of the proteins in the feed ingredients. These, in turn, will allow us to identify appropriate guidelines for the production of feed ingredients and extruded diets with a higher quality of protein.

ACHIEVEMENTS TO DATE

Suitable analytical methods are now available for the determination of the chemical parameters under study. A preliminary calibration of NIR equipment in order to get predictive estimates of fishmeal digestibility has been achieved.
Laboratory equipment for the controlled heat treatment of the raw material for fishmeal manufacture has been constructed and is now working satisfactorily. This apparatus has been used for the production of the material which will be analysed by the developed methods to determine the kinetics of formation of D-aspartic acid and of conversion of SH groups to S-S bonds. Significant progress has been achieved in planning the subsequent steps of the project, in particular with regard to the production of feeds and the conduct of feeding trials with fish.
Amino acid utilisation, nitrogen excretion and protein accretion in teleosts: interspecific comparison

OBJECTIVES

The main objectives of the project are to reduce ammonia-nitrogen losses through optimising amino acid balance and to elucidate the mechanisms explaining interspecific variations in nitrogen excretion and utilisation in four different species of teleosts (trout, sea bass, sea bream and turbot).

While some data on freshwater continental species are available, the potential of nitrogen utilisation and the routes and rates of nitrogen excretion of marine species have not received as much attention as they deserve. To benefit from the relative advance in our knowledge on continental freshwater species, the three marine finfish species will be compared with rainbow trout. The importance of amino acid balance on the reduction of nitrogen (ammonia and urea) excretion and optimisation of nitrogen utilisation will be studied. Specific attention will be paid to arginine, an essential amino acid in fish having multiple metabolic roles.

Therefore, the project will consist of undertaking specific nutritional trials with the four different finfish species in order to:

• determine maintenance and growth requirement of protein and arginine;
• study the effects of dietary arginine supply in plant protein-rich diets;
• evaluate the effects of dietary supply of nucleic acids.

The studies with complementary approaches will be conducted in a comparative manner, using the same protocols and diets for:

• quantifying amino acid needs for maintenance and growth;
• monitoring ammonia and urea excretions;
• determining the activities of key enzymes of overall amino acid oxidation as well as urea synthesis;
• measuring protein accretion and degradation.

The data obtained will enable the optimisation of dietary formulation leading to improved protein yield in the different fish species. This interdisciplinary comparative approach will form the basis of work on a general model on protein utilisation and nitrogen excretion by fish, which will be of use in estimating the potentialities of other new candidate species or those grown under different culture conditions.