"PROBASS": IMPROVING PRODUCTION EFFICIENCY OF SEA BASS FARMING BY DEVELOPMENT OF METHODOLOGIES TO ELIMINATE ENVIRONMENTAL ANDROGENESIS (Q5RS-2000-31365; 2001-2004).

The ultimate goal of PROBASS was to understand the mechanisms regulating sex differentiation in cultured sea bass, in order to develop methodologies to minimize the proportion of males in cultured stocks. To accomplish this aim two main objectives were pursued:

-In the first objective we performed a temporal evaluation during the period encompassing sex differentiation, in order to understand which sex differentiation genes, key steroidogenic enzymes, sex-steroid receptors, sex-steroid hormones, brain and pituitary hormones or growth factors were involved in the regulation of sex differentiation in males and females.

-In the second objective we manipulated the environment (temperature and density) during critical periods in early development, in order to reduce or eliminate male dominance in cultured sea bass, and to determine which regulators of the sex determination pathway were influenced by these conditions.

The **PROBASS** project consisted of four different parts:

The first part was directed to develop the necessary tools and methodologies for isolation of the mRNA sequences coding for candidate sex determining genes, steroidogenic enzymes essential for androgen/estrogen production, estrogen and androgen receptors, and insulin-like growth factors. These sequences and others already available within the consortium (GnRHs, GTHs and GH) allowed the development of sensitive RT-PCR methods to analyze mRNA expression of these proteins. In addition, specific inmmunoassay for brain, pituitary and growth factors were achieved to measure their tissue and plasma levels.

The second part consisted in the production of male- and female-dominant stocks of sea bass, taking advantage of the fact that from very early in development larger fish develop as females and smaller fish develop as males. From these populations morphometric data, blood and tissue samples were collected to examine the elements of the brain-pituitary-gonad axis mentioned above, at critical times prior, during an after completion of sex differentiation to detect sex-related changes in these elements.

The third part aimed to analyze the effect of environmental temperature and density on sex differentiation of sea bass. After performance of the experimental trials, sex ratio of the population was examined as well as the effect of the manipulation of these parameters on various factors potentially regulating sex differentiation and growth dimorphism in sea bass.

The fourth part took place during the last 15 moths of the project and included commercial trials to determine if fry produced with the developed temperature regime produced female-dominant stocks in industrial grow-out facilities

As a result of the work carried out in Workpackage (WP) 1 a large number of potential sex determining genes were cloned. These include DMRT-1, Dax-1,WT-1, Sox 3 and two isoforms of Sox 9 (together with 13 other members of the sox gene family). In addition, quantitative assays to determine levels of mRNA expression of some of these candidate sex determining genes were developed to examine their expression in response to size and sex, and after temperature or density manipulations (WPs 7 and 8). In WP2 full-length sequences for steroidogenic enzymes (brain P450 aromatase; P450aromB), steroid receptors (androgen receptor; AR, estrogen receptors ER□, ER□1 and ER□2), and gonadal markers (vasa) were obtained. The corresponding

semiquantitative RT-PCR assays for analysis of mRNA expression were set up, as well as ligand-binding assays for estrogen receptor and steroidogenic enzyme activity assays to measure aromatase activity. Furthermore, the promoter sequence from the ovarian aromatase gene was isolated and characterised. A number of promoter consensus sequences were found and gel retardation assays were used to confirm the authenticity of a putative SF-1 binding site. Most interestingly, three single nucleotide polymorphisms were observed within the promoter. These show linkage disequilibrium such that there appears to be two promoter alleles. In WP 3, quantitative PCR for GnRH forms, GnRHR and GP□, □FSH and □LH were developed to examine transcript levels in response to size and sex and after temperature manipulation. Works directed to the express recombinant GtH subunits were as well undertaken. Finally, we have established real-time fluorescence-based quantitative PCR assays for measuring transcript levels of growth hormone (GH) and insulin-like growth factors (IGFs), as well as a sensitive competitive ELISA for measuring GH. Through the work carried out in WP4, the project has confirmed the presence of sexual dimorphism in growth from a very early stage (about 2 months after hatching) in the European sea bass, favouring the female individuals, and has demonstrated the involvement of estrogens in the process of sex differentiation in the female. Through the work carried out in WP5, the project has demonstrated the involvement of not only genetics, but also environmental conditions (specifically temperature during larval rearing) on the sex ratio of the produced populations, and on the morphology and further growth of the fish. These data are very important from a physiological point of view, as they constitute the most thorough study to date on the European sea bass, in regards to this subject. Such knowledge can be useful to aquaculture producers, in terms of managing their stocks and planning their production cycles. Research in relation to the effects of rearing density on sex differentiation was undertaken in WP6. The results obtained, although counterintuitive, considering possible effects of density related stress, indicate that in the first two months after hatching sea bass environmental influences can modify sex. The purpose of WPs 7 and 8 was to analyze expression of candidate sex determining genes, steroidogenic enzymes, steroid receptors, regulators of aromatase transcription, and brain and growth factors and hormone levels during development. The effect of grading and temperature was also studied. Sex determining genes were identified that were expression is upregulated in either the developing male gonad (DMRT-1 and Sox9-2) or the developing female gonad (Sox31-1). The expression of both brain and ovarian aromatase forms were higher in females. In addition, it was suggested that polymorphisms in the aromatase promoter may be a factor in differential response of different groups of sea bass to altered temperature regimes. ER□ was highly expressed in female liver and pituitary of adult sea bass. In contrast expression of sb-ER□1 and sb-ER□2 was more wide-spread, with similar levels between tissues. During development, differences between timepoint of the expression peaks of the three ERs could be observed between female and male dominant populations. This could signify particular key functions of ERs during distinct periods of sexual differentiation and gonadal development. Very low levels of AR could be detected during early development for both experimental groups. No differences were observed between sexes at any sampling point. Nevertheless, considerably higher values were found in males during late development. The analysis of brain GnRH expression and pituitary profiles revealed that both sbGnRH and sGnRH were related with sex differentiation and changed according the predominate sex of the population analyzed. Gonadotropin subunits gene expression was correlated with those of sb and sGnRH in both S and L-extreme populations. In addition at the peak levels, the expression of FSHß was higher that that of LHß, indicating a higher implication of FSH than LH in the process o sex differentiation of sea bass. The mRNA levels and circulating concentrations of GH and IGFs were determined in two size-graded sea bass groups. IGF-I transcript levels were consistently higher (2-fold) than those of IGF-II, suggesting a more important role for

IGF-I during this age. Hepatic IGF-I mRNA levels fluctuated in correlation with the circulating levels of GH in both groups. This association attests to the direct role of GH in regulating hepatic IGF-I synthesis. Regarding sexual steroids it was observed that estrogens were associated with the differentiation of the female phenotype, but androgens exhibited an increase only during the process of precocious maturation in the males. Finally on-growing of female-dominant stocks of sea bass in commercial aquaculture facilities were undertake in WP9. This work was undertaken in collaboration with two industrial sub-contractors. Fingerlings were produced using the optimized thermal protocol (WP5) for the increase of female percentage in the population, and reared in marine cages, side-by-side with a similar-age stock produced under common industry methods. The growth and sex ratio of the two populations were examined at the end of the first year of growth, and was found that fish reared during the larval stage to lower temperatures had significantly higher percentage females in the population. In the case of the SE strain, the long exposure to low temperature is associated with a continuous suppression of growth beyond the treated period while, in the case of NW strain, the growth rate was similar among the two stocks.