Project Title**:** ***Towards the development of technologies for cryopreservation of fish oocytes (CRYOCYTE- Sept 2001- Nov 2005, Q5RS-2002-00784)***

Participating partners: E. Lubzens (Corodinator), ***H. Rosenfeld, I. Meiri,*** Israel Oceanographic and Limnological Research, Israel; ***P-E. Olsson***, Örebro University, Sweden; ***J. Cerdà***: Center of Aquaculture-IRTA, Spain***; P. J. Babin***, INRA, Université Bordeaux 1, France; ***A. Admon***, Technion, Israel; ***O. Carnevali***, Universita' di Ancona, Italy. ***D. M Rawson***, ***T. Zhang***, University of Luton, UK. ***M. Dichtl***, Carborus Metalicos, S.A., Spain; ***G. Pagelson***, Ardag Red Sea Mariculture, Israel

Project Objectives:

The main objectives are to develop methods for cryopreservation of fish oocytes while ensuring their viability after cryogenic storage and thawing. These aims will be achieved by studies conducted on: (**1**) Anticipated biological barriers for cryopreservation including the formation and structure of the vitelline envelope proteins and the process of oocyte hydration of pelagic eggs; (**2**) Identification of specific biological markers to monitor oocyte viability after manipulation and /or cryopreservation: (**3**) Development of oocyte in vitro incubation procedures to promote oocyte maturation, ovulation and fertilization: (**4**) Development of new cryopreservation technologies. Studies on two models [zebrafish (ZF) and the gilthead seabream (GSB)] highlight differences between marine and freshwater species and hydrating and non-hydrating oocytes. The results will improve fish production and increase its efficiency by genome banking of cultured and wild species.

Achievements:

1. Important steps were made in resolving for the first time the eggshell structure of ZF and GSB oocytes. The relative abundance of different zona pellucida (ZP) proteins was determined for eggshell proteins for ZF and GSB. Studies were performed to determine the localization of the four ZP proteins in ZF and GSB, by using fluorescently labeled specific antibodies. The results show that the ZPs have different patterns of deposition in the vitelline envelope and a new model has been proposed for their spatial distribution. The ZP proteins in GSB show different synthesis, regulation and deposition than those of ZF. The functional domains, important for polymerization and localization into the eggshell seem to be conserved among fish and are also present in amphibians, birds and mammals.

2. Molecular characterization of aquaporins in GSB and other teleosts has demonstrated that the GSB AQP1 belongs to a unique subfamily of AQP1-like aquaporins evolved in teleosts. At least one members of this novel subfamily, the SaAQP1o, is predominantly expressed in the ovary unlike any other vertebrate aquaporin described to date. The study has uncovered for the first time, the molecular mechanisms underlying the hydration of GSB oocytes, which is a highly controlled mechanism based on the interplay between protein hydrolysis and SaAQP1o, thereby illustrating a completely new and unexpected role for these proteins. The SaAQP1o peptides were localized in the cortical cytoplasm of early vitellogenic oocytes and as oocytes developed, was translocated towards a more peripheral area. During oocyte maturation, SaAQP1o was translocated into the oocyte microvilli, where it can potentially mediate water influx into the oocyte.

3. A serial analysis of gene expression (SAGE) was carried out in parallel with proteomic analysis on fully-grown ovarian follicles from ZF. Sequencing of 27,486 SAGE tags identified 11,399 different ones, including 3,329 tags with an occurrence

superior to one. Comparison with the large–scale expressed sequence tags sequencing approach revealed highly expressed transcripts that were not previously known to be expressed at high levels in fish ovaries. Comparison of transcriptome and proteome data revealed that transcript levels provide little predictive value with respect to the extent of protein abundance. This study provides a complete sequence data set of maternal mRNA stored in zebrafish germ cells at the end of oogenesis. The catalog contains highly expressed transcripts that are part of a vertebrate ovarian expressed gene signature. Comparison of transcriptome and proteome data identified downregulated transcripts or proteins potentially incorporated in the oocyte by endocytosis. The molecular phenotype described provides groundwork for future experimental approaches aimed at identifying functionally important stored maternal transcripts and proteins involved in oogenesis and early stages of embryo development.

4. Determination of the patterns of protein expression at different developmental stages of the oocytes using proteomics technologies was performed for ZF and the GSB oocytes. Analyses of protein profiles revealed that completely unique stage-specific proteins are likely not present in these oocytes and more likely the vast majority of the proteins are expressed in all stages of oocyte development, excluding the vitellogenin, which appears at Stage 3 and its pattern of processing can be used as a marker of early-vitellogenic oocyte. The large abundance of vitellogenin in the late stages of oocyte development masked and complicated the identification of other proteins. Proteins were identified from GSB (113 proteins identified by MudPIT and 2-DE and 22 proteins by 2-DE) and ZF (210 identified proteins) staged oocytes and the proteins patterns were established for each stage. The full identify of GSB proteins awaits the availability of gene sequences from this species

5. Studies on putative yolk processing enzymes from ZF and GSB ovaries was performed and included expression studies for Cathepsins B, D and L in different size oocyte as an indicator for their activity at various stages of vitellogenesis. Enzymatic assays were developed, improved and optimized to test for the activity of the cathepsins during vitelloegensis. The results indicated differences in the expression pattern during vitellogenesis within each examined species and also differences between ZF and GSB oocytes. In addition, morphological and ultrastructural studies of GSB and ZF oocytes during the processing of yolk proteins during oocyte maturation,were initiated. In general, these studies will contribute to discerning the role of yolk processing in maturation of oocytes and in the massive hydration observed in GSB oocytes, and for assessing viability in oocytes.

6. In order to promote *in vitro* final ovarian maturation (FOM), a recombinant GSB-LH (rGSB-LH) was successfully produced in the methylotrophic yeast *Pichia pastoris* expression system. *In vitro* bioassays, utilizing GSB ovarian follicles, indicated that the recombinant hormone is biologically active (at physiological plasma level concentrations) in inducing E_2 release as well as promoting FOM.

7. Studies were performed on cryopreservation of ZF and GSB oocytes by testing toxicity of cryoprotectant agents (CPAs), permeation to CPAs and cooling rates. The best results obtained so far of survival after cryopreservation with ZF oocytes were 16.3% using controlled slow cooling and 11.1% with vitrification by trypan blue staining. However, no indications of oocytes survivals were observed after cryopreservation when GVBD observation or ATP assessment were used. For GSB, small size oocytes (200-300μm) were found to survive (more than ~50%) cryopreservation procedures as evaluated by MTT staining and morphological examination, but not larger ones. The main apparent obstacle seems to be a change in the physical structure of vitellogenin induced by ice-nucleation.