Project Proposal

My future research addresses: Molecular mechanisms of genetic (natural) and environmental sex differentiation in fish using the valuable aquaculture tilapia cichlid species, *Oreochromis niloticus*. This work will be focussed on profile changes in both the brain and the gonads.
My proposed research includes 3 specific aims designed to test the hypothesis that temperature or hormone driven sex differentiation follows a similar cascade to that of male or female genetic sex differentiation.

Aim 1: Identify genes associated with natural sex differentiation by comparing expression profiles for developing male and female gonads using a brain microarray from the cichlid *Astatotilapia burtoni*, hybridized with RNA from tilapia *O. niloticus*.

Aim 2: Environmental sex differentiation will be analysed by comparing expression profiles of temperature-induced males, and oestrogen-induced females *versus* natural development.

Aim 3: To analyse the respective role of the central nervous system (brain) and gonads during the natural or induced (temperature and oestrogen) sex differentiation by comparing the kinetic expression profiles in these two tissues.

The JEB International Travel Fellowship will allow me to complete **Aim 1** and initiate **Aim 2**. This work will foster a collaborative project between myself, Dr. Helena D'Cotta and Dr. JF Baroiller from the Aquaculture Unit at CIRAD, France, and Dr. Susan Renn and Dr. Hans Hofmann at Harvard University's Bauer Center for Genomics Research, USA. This collaboration will provide me with the opportunity to enhance my knowledge concerning microarray production involving novel experimental designs, hybridizations, as well as the analysis of microarray data, which I can then bring to the CIRAD functional genomics initiative at Biotrop to expedite the advancement of our Aquaculture studies at CIRAD as well as to the Hanoi University of technology in Vietnam, where I will work after the completion of my post-doctoral position at the Aquaculture Unit of CIRAD.

Background and Significance

In tilapia sex is determined by major genetic factors located on sex chromosomes, minor modifiers located on the autosomes, rearing temperature during the critical period of sex differentiation and by the interaction between these genetic and environmental factors (Baroiller *et al*., 1999; Baroiller and D'Cotta, 2001). The co-existence of major genetic factors on sex chromosomes similar to mammals as well as environmental influences, similar to what is found in amphibians and reptiles, makes the tilapia an original model to study sex differentiation. As temperature can completely override the genetic sex determination (Baroiller *et al*., 1995), an exciting challenge is to test the hypothesis that sex differentiation follows a similar path under natural or temperature-induced conditions.

Studies of the molecular mechanisms for sex differentiation in fishes have been focused on a small number of fish species (medaka, trout and tilapia), in which genetic all-males or all-females populations (respectively produced from YY males or XX males) are available. These studies, mainly based on candidate gene approaches, have clearly demonstrated the key role of steroids and particularly estrogens in female differentiation (Baroiller et al. 1999, Devlin and Nagahama, 2002). However, steroids are not the inducers of sex differentiation in fish, and other genes such as *Dmrt1* play a major role in testes differentiation (Marchand *et al*., 2000; Devlin and Nagahama, 2002). Molecular mechanisms of the temperature-induced sex differentiation have been only studied in 2 species, the tilapia, *O. niloticus* and the Japanese hirame, *Paralichthys olivaceus*. Here again, these candidate gene studies have demonstrated the important role of estrogens in the temperature effects on sex differentiation (D'Cotta et al, 2001a, b; Kitano et al., 2001). More exhaustive approaches, suggest that temperature can both up and down-regulate the expression of some genes implicated in the cascade of sex differentiation. Due to the complexity of the molecular mechanisms involved in sex differentiation, exhaustive approaches, such as functional genomics, are required in order to better understand these mechanisms.

Finally, a better knowledge of sex determination and its subsequent control constitutes a major challenge for fish farming in view of the several-fold benefits of producing mono-sex

populations (growth, flesh quality or susceptibility towards disease). In tilapias (second most important World group of freshwater fish, 1.5 million tons/year), male monosex populations are the solution to face the problem of uncontrolled reproduction and to benefit of the fast growth-rate of males. Currently all-male populations are mainly produced through hormone treatments applied before and during the process of sex differentiation. Although in principle ecologically compatible, genetic approaches have so far met with only moderate success (i.e. the use of YY males). A better understanding of sex determination and sex differentiation should allow the production of all-male populations by environmental manipulations, or by selection for the major or the minor genetic factors.

The **CIRAD research group** "Biology & Improvement of Fish Species for Aquaculture" from the Aquaculture Unit, has been working since 1985 on sex determination and sex differentiation, primordially in the cichlid tilapia species. Following our first demonstration that temperature can influence sex differentiation in tilapia (Baroiller et al., 1995), a particular emphasis has been made on environmental sex determination in this species. In 1997 the group began developing molecular approaches to search for differentially expressed transcripts under genetic (natural) and temperature-induced gonadal sex differentiation (D'Cotta et al., 2001a, b). We are currently generating a gonad macroarray which will contain a minimum of 2000 clones, enabling the study of genotypic and phenotypic sex profiles from tilapia gonad.

The Hofmann laboratory at Harvard University's Bauer Center for Genomics Research has constructed a cDNA microarray with ~ 4500 features from a brain-specific cDNA library for the the Tanganyikan cichlid, *A. burtoni* which is closely related to the focal species of this study (~10 MY). The incredibly recent radiation of this group has resulted in diverse phenotypes with little genomic divergence this making it an excellent model system with which to investigate the evolution gene expression patterns underlying complex phenotypes through a comparative approach. The Hofmann laboratory has quantified the utility of their microarray to study expression profiling in other fish species through heterologous hybridization (Renn et al, 2004). They have directly tested the tilapia species, central to the proposed project (see below)

The Cichlid Consortium, to which both research groups contribute**,** has recently submitted a Whitepaper proposal to sequence the genome of Nile tilapia, the cichlid where many genomic tools exist (BACs, genetic mapping and genes cloned). Taking advantage of the low genomic divergence among these fish, the cichlid consortium intends to expand genomic tools (e.g. microarrays) that will be applicable to many species. In this way, even without a completed genome sequence, the field of genomics can be applied to enhance the understanding of complex traits in cichlids and applied to interesting physiological, evolutionary, and ecological questions of cichlids.

Methods and Design

Because of the plasticity of sex differentiation in fish, steroid and temperature treatments can induce a complete functional sex inversion to occur and hence, allow the production of important genetically all-female populations, and of genetically all-male populations of genotypes XX, XY or YY. Such populations allow early sampling of identified genotypes and future phenotypes, before the appearance of any signs of histological sex differentiation.

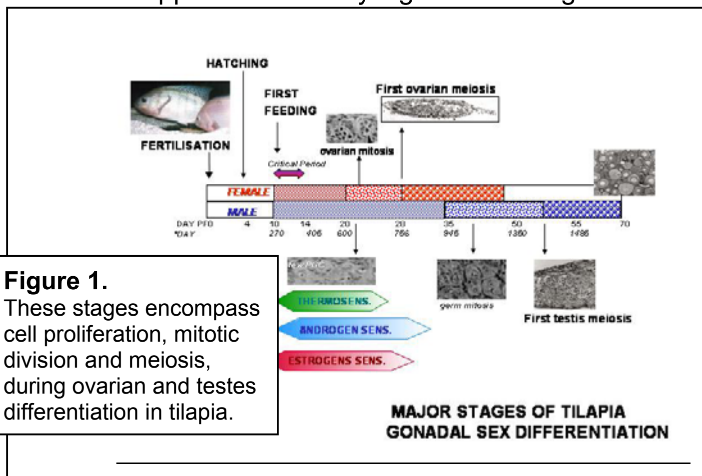

Figure 1.
These stages encompass cell proliferation, mitotic division and meiosis, during ovarian and testes differentiation in tilapia.

A sampling of the gonad and brain tissue has been performed throughout the three key stages of tilapia sex differentiation (Fig. 1) for genetic all-male and all-female individuals. Furthermore, I have tissue available for RNA extraction of experimentally induced sex-differentiation of both hormonally manipulated and temperature altered populations from these same lines. RNA will be labelled according to a standard procedure of indirect labelling through reverse transcription incorporation of Amino-allyl dUTP that is then

coupled to the appropriate Cy-dye fluorophore.

While the proposed project extends current microarray practice by relying on heterologous hybridization, (hybridizing samples from a species other than that from which the array platform was constructed, e.g. primate on human array [Enard et al., 2003] squirrel on rat array [Hittle and Storey 2001]), the scope of this project is limited to within species comparisons of tilapia developmental stages therefore species bias will not compromise the accuracy of the results and conclusions. Nonetheless, interspecies genomic hybridization will be used as a control to determine those spots which will provide the most reliable data (Fig 2, Renn et al., 2004). Because a large percentage of the genome protein-coding genes are expressed in the brain (Datson et al., 2001; Evans et al., 2002), this microarray, derived from a brain cDNA library will also be useful for expression profiling in other tissues. In fact preliminary experiments competitively hybridizing RNA from ovaries and testes of another cichlid species demonstrate that the majority of the spots on the array represent genes that are expressed at detectable levels in mature gonads (91% ovary, 77% testes) (data not shown).

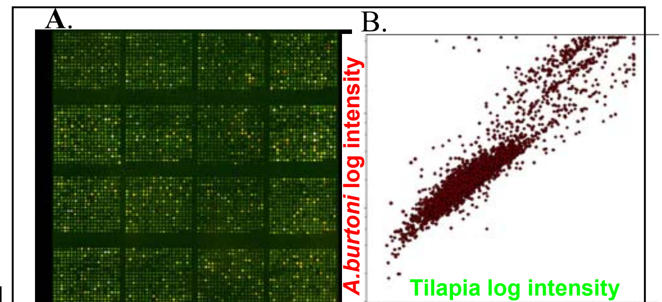

Figure 2 Competitive Genomic hybridization A) array image of *A.burtoni* (red) and *O. niloticus* (green) B) median intensity is approximately equal (yellow un-normalized ratio) for both channels for the majority of the spots demonstrating that they will be usable for intra-species hybridization of RNA samples from *O. niloticus*

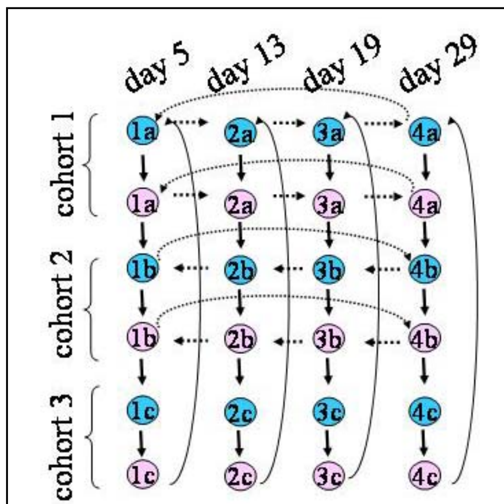

Figure 3. Loop design for natural sex differentiation in tilapia days indicate significant developmental timepoints. 3 independent cohorts will control for variation. arrows indicate direct hybridization (tail=Cy3; head=Cy5)

Many recent reviews discuss the fundamental issues experimental design (e.g. Churchill 2002). For this study I propose to use a loop design with each condition represented 4 times. This design includes dye-swaps for each of the 2 between sex within stage and the two within sex between stage comparisons) Although not all direct comparisons of sex and time-point occur together on a single array, comparisons between all pairs can be made indirectly by utilizing the cyclic nature of the loop design. While this does result in increased comparison-wise variance, the effect is minimal for small loops.

This balanced design, including dye swaps, yields data which can be optimally analyzed by Bayesian analysis of Gene Expression Levels (BAGEL Townsend & Hartl, 2002), which is robust to missing data (individual spot defects, and failures) and powerful for identifying statistically significant gene expression difference even for small fold changes. Form normalization and ANOVA based analyses, the open source software packages designed for Bioconductor will be used (R-language http://www.bioconductor.org/).

Future Collaboration:

My collaboration with the Hofmann lab will increase the search for sex differentiating genes by permitting the analysis of sex profiles in the brain without the requirement of creating a tilapia brain cDNA library and microarray. The brain seems to play a major role in tilapia sex differentiation with expression of important sex differentiating transcripts (D’Cotta et al., 2001a; D’Cotta unpublished data). The knowledge of microarray technique, experimental design, and analysis I will acquire while at Harvard will enable me to identify novel sex-differentiating genes; it will also be instrumental to the development of genomic resources as part of my work with the CIRAD Aquaculture team and later at the Hanoi University of Technology.

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