

Functional genomics and quantitative PCR for the study of environmentally related gene expression in *Pseudopleuronectes americanus*

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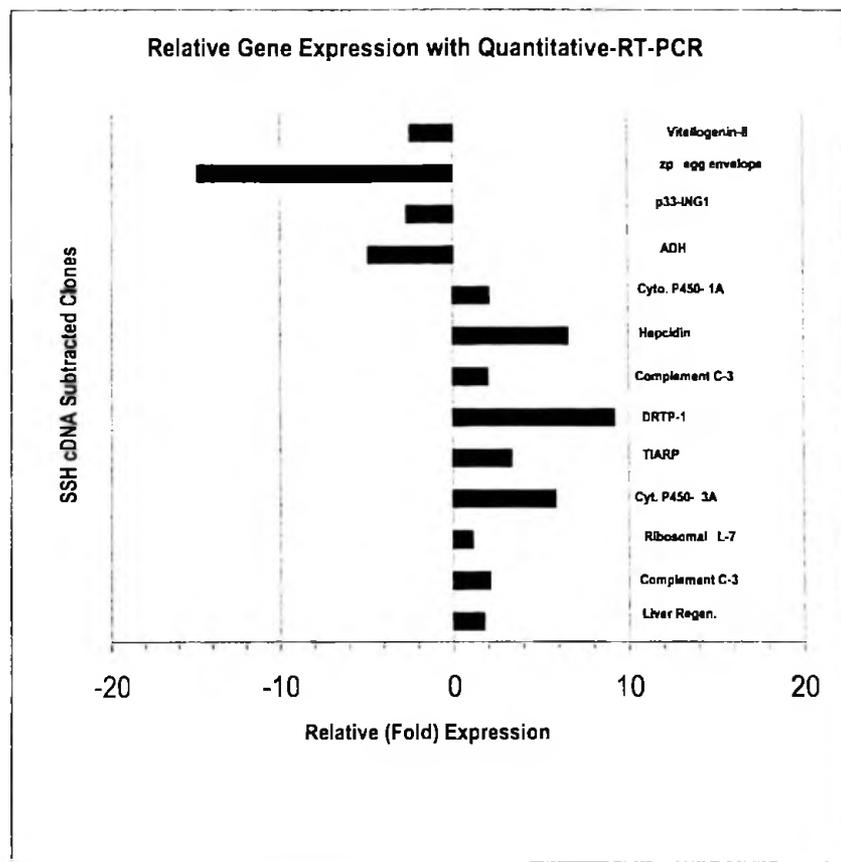
Winter flounder (*Pseudopleuronectes americanus*) is an excellent model system to detect the transcriptional response of vertebrate organisms to chronic environmental exposure to sub-lethal levels of contamination in marine waters and sediments. Winter flounder migrate each fall into back-bays and estuaries, remaining through the winter and leave for deeper water in the spring after spawning. During this period, they may be exposed to high levels of anthropogenic wastes, particularly in urbanized estuaries such as the Hudson-Raritan-New York Harbor and Boston Harbor. It is well documented that environmental exposure in contaminated habitats results in a high incidence of cellular changes in the fish liver including development of hydropic vacuolation, cirrhosis and neoplasias^{2,3}. Other problems noted are high incidence of fin rot, skin lesions, feeding depression and failure to reproduce². The goal of this study was to use transcriptome analysis to detect altered gene expression related to environmental condition in the winter flounder⁴. The first objective of this work was to isolate a large number of potentially environmentally responsive genes related to anthropogenic pollution. The second objective was to identify these genes by DNA sequencing and computer homology search. The third objective was to develop quantitative PCR assays for these isolated genes to ascertain if they were truly environmentally responsive.

Winter flounder were collected during their spring out-migration from the highly pollution-impacted Hudson-Raritan estuary in northern New Jersey and from less impacted sites in southern NJ. Total RNA was extracted from liver tissue, pooled from fish collected from each type of environment, using phenol and guanidine isothiocyanate. After the subsequent extraction of polyA mRNA, two suppression subtractive hybridization (SSH) PCR cDNA libraries were produced¹. The forward library consists of partial transcript cDNAs from genes that were up-regulated or more highly expressed in the pollution impacted fish. The cDNA in the reverse library represents genes that were down-regulated with pollution, that is, they were more highly expressed in the control animals. Individual cDNAs from the libraries were cloned and purified for sequencing. This sequence data was then used to search for homology with genes in GenBank[®] using the Blast algorithm at <<http://www.ncbi.nlm.nih.gov>>. PCR primers were designed for a set of putatively environmentally regulated genes for use in quantitative PCR to quantify their relative levels of expression in fish from both types of environments.

A total of 251 differentially expressed transcripts were isolated from the forward and reverse libraries made from winter flounder liver tissue by SSH PCR. Each was assigned a homology based on the results of a Blastx search and entered into the database of expressed sequence tags, dbEST, at GeneBank[®] (CF195396-647). About 65% of the cDNA sequences were putatively identified by the homology search. Functional categories for the transcripts identified included genes associated with immune response, metabolism, detoxification, growth factors, tumor suppression and reproduction. Some of these were: complement factors C-3, C-7, factor H, the antimicrobial peptide hepcidin, cytochrome P450 1A, cytochrome P450 3A, glutathione-s-transferase, tumor suppressor p33 ING1, hepatocyte growth factor protein, bal -643 liver regeneration protein, ceruloplasmin, vitellogenin's I & II and zona pellucida (zp) egg envelope protein. Quantitative PCR assays were developed for twelve of these putatively identified differentially regulated genes. The results of the quantitative PCR assays (Figure 1.) indicate that the qualitative differences seen in the forward (up-regulated with pollution) and reverse (down-regulated) libraries have a quantitative basis in the differential expression of these

genes. The up-regulation of immune responsive transcripts for hepcidin, complement C-3, and DRTP-1 (differentially regulated trout protein-1) may indicate high bacterial or viral loads. Higher expression levels of cytochrome P450 1A and 3A, indicate activation of liver detoxification genes. Decreased expression of zp egg envelope and vitellogenin transcripts suggest interference with reproduction. While increased levels of liver regeneration related protein and decreased levels of ADH and tumor suppressor p33ING1 may indicate damage to the liver tissue.

Figure 1. Relative expression by quantitative PCR of differentially expressed genes in fish from pollution-impacted waters vs. from less impacted waters. Positive relative expression values indicate fold induction with pollution; negative values indicate inhibition with pollution. Fold induction or inhibition is the ratio of sample template to respective control template. The genes tested were: vitellogenin II, zp (zona pellucida) egg envelope protein, p33ING1 (tumor suppressor), ADH (alcohol dehydrogenase), cytochrome P450-1A, Hepcidin (anti-microbial peptide), complement C-3 (region 2), DRTP-1 (differentially regulated trout protein-1), TIARP (tumor necrosis factor alpha induced adipose related protein), cytochrome P450-3A, Ribosomal protein L-7, Complement C-3 (region 1), Liver Regeneration Protein (Bal-643).



In addition to the negative impacts on fish, components of anthropogenic waste in urbanized estuaries pose a significant health hazard to human health, with DDT, dioxin, PCB's, heavy metals and partially processed sewage among the most dangerous. Biomagnification of dangerous compounds through the food web into the human diet can become a significant health risk. These studies serve two purposes, the first is to develop models in marine fish of the effects of chronic exposure to toxicants at the transcriptional level and the second is to develop effective assays to detect the effects of sub-lethal contamination in fish that may impact human health via dietary consumption.

This work was supported by a New Investigator Award from the MDIBL, Center for Membrane Toxicity Studies under NIEHS P30 ESO3828-18, a Richard Stockton College Distinguished Faculty Fellowship, the National Science Foundation under MRI #0116165 and REU #0097635 and the US Dept of Commerce/NOAA National Sea Grant #NA16RG1047. The views expressed do not necessarily reflect the views of these organizations. This is a publication of NJ Sea Grant-04-555.

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