WASHINGTON SEA GRANT PROGRESS REPORT
for the period 2/1/2008 – 1/31/2009

WSG Project Number: **R/B-49**
Project Title: Development of Genomic Biomarkers for Assessing Fish Reproductive Health

Principal Investigator(s) and Affiliation:
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1. ABSTRACT ELEMENTS

OBJECTIVES
This project has two long-term objectives. The first objective is to develop comprehensive bioassessment tools that can generate "molecular fingerprints" in the monitoring of impacts of environmental conditions and contaminants on the reproductive health of salmon. The second objective is to provide training for graduate and undergraduate students in advanced genomic technologies for assessing the physiological status of fish. We propose to characterize gonadal gene expression patterns that occur during early stages of gametogenesis in healthy fish compared to fish exposed to chemicals or environmental conditions that are known to inhibit fish reproduction. Early gametogenesis will be targeted because of its critical importance in influencing reproductive fitness characters (gamete numbers, age of maturity, fertility and embryo quality). Furthermore, these stages occur when salmon are migrating through or residing in urban waterways, estuaries, and nearshore environments where they are exposed to a variety of anthropogenic environmental factors. We will develop custom DNA microarrays to determine perturbations in gonadal gene expression in salmon exposed to natural and anthropogenic environmental factors.

METHODOLOGY
Coho salmon will be used as the experimental model because development of a single class of gametes is synchronous, which allows correlation of gene expression patterns with specific stages of gametogenesis. We plan to identify gonadal genes that are up- or down- regulated in response to environmental stressors by constructing and sequencing cDNA libraries from gonadal mRNA after suppression subtraction hybridization (SSH). During the project period we will focus on characterizing ovarian gene expression after two types of environmental stress that are known to alter gonadal growth: feed restriction and xenoestrogen exposure. Later studies will test a third environmental stressor (hypoxia) and examine testicular gene expression. In the first experiment, SSH will be done on samples from short (2 week) and long-term (6 week) fasted fish to capture molecular events in the ovary that both regulate and result from atresia (both precede and coincide with morphological signs of atresia). In the second experiment, SSH will be done on ovarian samples from fish exposed to ethynylestradiol via tank water for six weeks. In both studies, we will perform SSH on mRNA from both whole ovarian and follicle-enriched tissue. The effects of experimental treatments on large-scale ovarian gene expression will be determined by quantitative real-time RT-PCR assays of ovarian genes identified from the SSH.

Custom oligonucleotide microarrays will be designed using genes identified from these studies and relevant genes selected from exisiting EST databases for fish ovaries and testes. These microarrays will later be modified as new ESTs appear in the databases, and we complete tests of other environmental stresses and studies of testis gene expression.

RATIONALE

Urbanization, coastal population growth, agricultural practices and climate change have significantly altered water quality throughout aquatic ecosystems of the Pacific Northwest Region. Many chemicals contaminating fish and aquatic ecosystems are capable of disrupting endocrine function leading to impaired development and reproduction of both humans and wildlife. Yet, the long-term sublethal effects to fish and humans that consume them are poorly understood. Efforts to restore and enhance fish stocks could be significantly undermined if contaminants, or other environmental conditions, in critical habitat impair reproductive health. Therefore, comprehensive bioassessment tools are needed for fisheries researchers to evaluate relationships between specific environmental conditions/contaminants and reproductive health of fish. Global assessment of gene expression is more advantageous than studies surveying a few genes or single biomarkers because if a sufficiently diverse set of genes is monitored, toxicant and mode of action-specific responses can be identified and used as a molecular fingerprint for environmental monitoring. This project is appropriate for Sea Grant because it will develop advanced biotechnological tools for monitoring fish health that will aid efforts to understand: 1) interactions among marine resources, ecosystem health and human activities, and 2) impacts of factors that affect success of stock restoration and enhancement. It will also provide graduate students with training needed to use genomic technologies in marine research.

2. ACCOMPLISHMENTS AND OUTCOMES

During the second year of the project we completed the first set of subtractive hybridization (SSH) cloning to identify differentially expressed genes in healthy versus unhealthy ovaries of coho salmon that were subjected to long term fasting to induce an unhealthy state in the ovary (atresia). This included cloning, sequencing, bioinformatics analyses to characterize the cDNA libraries and identify genes based on homology to known genes in other species, and real time RT PCR to quantify the changes in transcripts at various time points during the fasting period. We were interested in identifying genes that were up- or down-regulated at early phases of ovarian regression, prior to morphological signs of degeneration of oocytes as well as genes that were expressed when atresia was advanced. After 14 weeks of fasting, the ovaries of fasted fish contained a mixture of unhealthy (atretic) and healthy oocytes that were at a similar stage, but smaller than those of the fed fish. In the SSH libraries from samples collected at 14 weeks post-fasting we identified 304 genes that were down-regulated and 319 genes that were up-regulated in the ovaries from fasted fish that clearly had initiated atresia compared to those of fed fish that had primarily healthy oocytes. About 28% of these genes were identified based on homology to known genes and 72% were categorized as unknown. Genes that were up-regulated in the normally growing ovaries from fed fish included genes involved in lipid transport and accumulation, yolk processing and steroidogenesis. In contrast, up-regulated genes in the ovarian library from fasted fish included those involved in apoptosis (programmed cell death) as well as components of the zona pellucida and cortical granules, which is probably due to the fact

that the ovaries from fasted fish had a mixture of atretic and healthy oocytes. Based on analysis of transcripts for apoptosis by quantitative real time RT PCR, we determined that the primary pathway of apoptosis was that of the Fas ligand-FADD-caspase 3 because transcripts for all of these were elevated at 9 weeks post fasting, well before morphological signs of atresia. In addition, transcripts for several steroidogenic enzymes were depressed at 9 weeks post fasting, prior to any decline in plasma estradiol levels. These data indicate that molecular markers indicative of atresia and reduced steroid biosynthesis could be used to identify ovarian follicles that were likely to be unhealthy and initiating atresia well in advance in changes in ovarian morphology or plasma hormone levels. We are continuing to analyze expression of other genes identified from the SSH libraries and libraries produced from ovarian follicle cell enriched preparations.

3. IMPACTS

Several molecular markers of advanced stages of programmed cell death in salmon tissues were identified, and we will proceed with the large scale cloning to identify other molecular pathways that are activated early in the process of ovarian atresia. The markers we currently have could be useful tools for monitoring the process of cell death in salmon under experimental conditions that could be detrimental to normal tissue growth in salmon.

4. PERFORMANCE MEASURES

Measure 1: Economic and societal benefits derived from the discovery and application of new sustainable coastal, ocean, and Great Lakes products from the sea.

Actual (reporting period covered by this report):

Anticipated (12-month period following this reporting period):

Measure 2: Cumulative number of coastal, marine, and Great Lakes issue-based forecast capabilities developed and used for management.

Actual (reporting period covered by this report):

Anticipated (12-month period following this reporting period):

Measure 3: Percentage/number of tools, technologies, and information services that are used by managers (NOAA and/or its partners and customers) to improve ecosystem-based management.

Actual (reporting period covered by this report):

At least fifteen gene transcripts were identified that could be used to monitor health of the ovary of salmon. These genomic markers could be applied to monitoring effects of habitat quality (water quality, temperature, etc.) on reproductive status of female salmon or trout.

Anticipated (12-month period following this reporting period):
We anticipate identifying additional genomic markers of acute versus chronic exposure to environmental estrogens using subtractive hybridization cloning.

5. PUBLICATIONS

Please refer to instructions for hardcopy reprint requirements and citation formats.
None to date. One paper in preparation

6. PRESENTATIONS - inc. Conference (Poster or Oral), Seminar & Public

Conference poster presentation- Yamamoto Y, Luckenbach, JA, Goetz, FW, Young, G and Swanson, P (2009) Disruption of the salmon reproductive endocrine axis through prolonged nutritional stress: changes in circulating hormone levels and identification of differentially expressed ovarian genes. Poster presentation at the Annual Meeting of the Society for Study of Reproduction, Pittsburgh, PA July 21-24.

Symposium poster presentation- Yamamoto Y, Luckenbach, JA, Goetz, FW, Young, G and Swanson, P (2009) Genomic biomarkers for assessing fish reproductive health. Poster presentation at the Northwest Fisheries Science Center Symposium, February 25-26, 2009.

Symposium oral presentation. Swanson, P, Luckenbach, JA, Yamamoto, Y, Dickey, JT, Beckman B, Larsen D, Cooper K, Felli L, Goetz FW, and Young G. (2009) Canaries in the Sea-monitoring physiological status of fish in the context of the environment. Oral presentation at Northwest Fisheries Science Center Symposium, February 25-26.

7. PATENTS AND COPYRIGHTS

8. NEW BUSINESSES OR JOBS CREATED

9. LIST ALL STUDENTS SUPPORTED BY OR AFFILIATED WITH THIS PROJECT

___ no students involved (check here if no students were involved in the project)

We presently have one undergraduate research student involved in the project and have recruited a graduate student who will start in Fall 2009.

Student Name: Molly Middleton
Department: School of Aquatic and Fishery Sciences
Major/Degree field: Fisheries
Major Professor:
Student Type (Ph.D., M.S., M.A., B.S., B.A. J.D., etc): BS, undergraduate
Dissertation/Thesis title:
Date of graduation (actual or anticipated): 2010
Total support or affiliation period (e.g., Jan – June 2005): April 2008- present
Type of support (RA, research costs, conferences – list all that apply): research costs
Current employment if applicable:

10. INTERACTIONS

none to date

11. OUTREACH AND INFORMATION/TECHNOLOGY TRANSFER

We currently have a volunteer from the public, Korin Keyser, who is assisting with aspects of the project to learn more about environmental physiology of fish and applications of this science to restoration of ecosystems such as Puget Sound. She received her degree in Biology from University of Washington and has been working in the biotechnology area. Since working with us, she has become interested in pursuing a higher degree.

We have submitted cDNA sequences of genes expressed in the ovary that we identify in this project to GENBANK. This makes all genomic information available to the wider scientific community.

12. FUTURE ACTIVITIES

During the coming year, activities will be directed toward completing the identification and quantification of genes up- or down- regulated in the ovary after long term fasting and submission of manuscripts describing results of this work for publication. We will also initiate a study to identify genes that are up- or down-regulated in the coho salmon ovarian follicle cells and whole ovary after exposure to an environmental estrogen (ethynylestradiol, EE2). This will be done in summer and fall 2009 on 1+ age coho salmon. We have recruited a graduate student, Louisa Harding, who will start in fall 2009 pursuing a MS in the School of Aquatic and Fishery Sciences, University of Washington. She will conduct parts of this study as a graduate research project. Our plan is to use subtractive hybridization cloning (SSH) as outlined in our proposal. However, as part of another project we are exploring the use of the next generation DNA sequencing technology and an in silico subtraction method. The advantage of this method is the large number of sequences that can be obtained in a single run (millions of 30-40 base pair fragments). If this method proves to be advantageous and cost effective we may use this instead of the SSH. It is anticipated that completion of the EE2 exposure study and conducting the SSH cloning, sequencing and bioinformatic analyses of the data, and transcript measurements will

take 12-15 months. As genes are identified from the SSH, quantitative real time RT-PCR assays will be developed to validate the results of the SSH and to examine how transcript levels change in samples collected from the EE2 exposure study. From these data we will be able to determine how the patterns of ovarian gene expression vary after acute versus chronic exposure to EE2. During the coming year, we will also assemble an EST database for the salmon ovary based our results and begin to identify genes that are candidates for a custom cDNA array for salmon ovarian genes.