Exxon Valdez Oil Spill
Restoration Project Final Report

Laboratory Examination of Oil-Related Embryo Mortalities that Persist in Pink Salmon Populations in Prince William Sound

Restoration Project 97191A-2
Final Report

James E. Seeb
Christopher Habicht

Alaska Department of Fish and Game
Genetics Laboratory
333 Raspberry Road
Anchorage, AK 99518-1599

May 1999

Laboratory Examination of Oil-Related Embryo Mortalities that Persist in Pink Salmon Populations in Prince William Sound

Restoration Project 97191A-2
Final Report

Study History: The molecular genetics component of Restoration Project 97191A, the study of persistent mortality of pink salmon embryos, was initiated in 1992 as Restoration Study 60C. Project 60C and the 9x191 series of projects were corollary to studies originating in March 1989 and continuing through February 1991 as Fish/Shellfish Study Number 2. That project consisted of embryo sampling in the fall and pre-emergent fry sampling in the spring at oil-contaminated streams and non-contaminated reference streams to determine if the *Exxon Valdez* oil spill affected pink salmon incubating in the oiled gravel. In 1992 the field sampling was expanded to include (1) laboratory evaluation of field results through the controlled incubation of pink salmon embryos on oiled substrate (NOAA); (2) an experiment designed to determine if the results observed in the field were due to environmental factors (ADF&G); and (3) a search for evidence of genetic damage (ADF&G). This work continued as Restoration Project 93003 and as the Restoration Project 9x191 series. Final reports were completed for Fish/Shellfish Study 2, Restoration Study Number 60C, and Restoration Project 93003 (all entitled Injury to Salmon Eggs and Preemergent Fry in Prince William Sound). An annual report was submitted for Restoration Project 94191 (Injury to Salmon Embryos and Preemergent Fry in Prince William Sound). Since then annual reports were separated for the field component (9x191A-1) and laboratory component (9x191A-2). This is the final report for the laboratory component of 97191A which focused on genetic screens for genetic damage. The primary unreported element at the time of closeout was a subcontract to NYU Medical School to examine potential mutations in the K-*ras* oncogene in response to exposure to crude oil. Funds from this project series funded parts of the ADFG research reported in components of the following publications:

Miller, G.D., J.E. Seeb, B.G. Bue, and S. Sharr. 1994. Saltwater exposure at fertilization induces ploidy alterations in salmonids. Can. J. Fish. Aquat. Sci. 51:42-49.

Greene, B.A., and J.E. Seeb. 1997. SINE and transposon sequences generate high-resolution DNA fingerprints, "SINE prints" which exhibit faithful Mendelian inheritance in pink salmon *(Oncorhynchus gorbuscha)*. Mol. Mar. Biol. Biotech. 6(4): 331-341.

Seeb, J.E., C. Habicht, J.B. Olsen, P. Bentzen, J.B. Shaklee, and L.W. Seeb. 1998. Allozyme, mtDNA, and microsatellite variants describe structure of populations of pink and sockeye salmon in Alaska. Pages 300-318 in D. Welch, D. Eggers, K Wakabayashi, and V. Karpenko (*eds.*) Assessment and Status of Pacific Rim Salmonid Stocks. North Pacific Anadromous Fish Commission, Bulletin No. 1, Vancouver, Canada. 513p.

Bue, B.G., S. Sharr, and J.E. Seeb. 1998. Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the *Exxon Valdez* oil spill. Trans. Amer. Fish. Soc. 127: 35-43.

Olsen, J.B., P. Bentzen, and J.E. Seeb. 1998. Characterization of seven microsatellite loci derived from pink salmon. Molecular Ecology 7(8):1087-1089.

Habicht, C., S. Sharr, D. Evans, and J.E. Seeb. 1998. Coded wire tag placement affects homing ability of pink salmon. Trans. Am. Fish. Soc. 127(4): 652-657.

Roy, N.K., J. Stabile, J.E. Seeb, C. Habicht, and I. Wirgin. 1999. High frequency of K-*ras* mutations observed in pink salmon embryos experimentally exposed to *Exxon Valdez* crude oil. Environ. Toxicology and Chemistry 18(7):77-84.

Spruell, P., K.L. Pilgrim, B.A. Greene, C. Habicht, K.L. Knudsen, K.R. Lindner, J.B. Olsen, G.K. Sage, J.E. Seeb, and F.W. Allendorf. 1999. Inheritance of nuclear DNA markers in gynogenetic haploid pink salmon. J. Heredity 90(2):289-296.

Abstract: We used an array of genetic detection methods to test the hypothesis that incubation of pink salmon embryos in an oiled substrate induces genetic damage. Mortality and abnormality rates were higher for embryos in oiled incubators than for those in unoiled incubators. Despite high statistical power, we found no relationship between exposure to oil and incidence of genetic damage detectable by flow cytometry. Androgens produced from males incubated in oil as embryos demonstrated no elevated mortality; however, this experiment lacked statistical power due to unanticipated male-to-male variability. We found unexpectedly little polymorphism and no differences in either cytochrome *b* or the region of tumor suppressor gene *p53* reported to be hypervariable in other species. A pilot study of four microsatellite loci showed no change of allelic expression. However, a high frequency of K-*ras* mutations was observed in pink salmon embryos experimentally exposed to *Exxon Valdez* crude oil.

Key Words: Cytochrome *b*, *Exxon Valdez* oil spill, flow cytometry, genetic damage, mtDNA, mutation, *Oncorhynchus gorbuscha*, pink salmon, Prince William Sound, *p53*, *Tc1/SmaI*.

Project Data:

Data	Software	Contact Person
Flow cytometry	Multicycle software	Chris Habicht
Mortality data	Excel spreadsheet	Chris Habicht
Haploid androgenesis	Excel spreadsheet	Gary Thorgaard
Cytochrome b and p*53* DNA sequence	Applied Biosystems, Inc. Sequence Analysis software	Eric Kretschmer
K-*ras* DNA sequence	Excel spreadsheet	Ike Wirgin

Citation:

Seeb, J.E., and C. Habicht. 1999. Laboratory examination of oil-related embryo mortalities that persist in pink salmon populations in Prince William Sound, *Exxon Valdez* Oil Spill Restoration Project Final Report (Restoration Project 97191A-2), Alaska Department of Fish and Game, Genetics Laboratory, Anchorage, Alaska.

TABLE OF CONTENTS

Page

LIST OF FIGURES 5
LIST OF TABLES 6
LIST OF APPENDICES 7
EXECUTIVE SUMMARY 8
INTRODUCTION 11
Flow Cytometry 13
Androgenesis 14
Development of Mutation Screens for Microlesions in Pink Salmon 14
Mitochondrial DNA 14
Tumor Suppressor Genes and Oncogenes 15
Microsatellites 15
DNA fingerprinting 15
OBJECTIVES 16
METHODS 16
Controlled Matings and Oilings 16
Flow Cytometry 17
Androgenesis 18
Polymerase-Chain-Reaction Based Screens for Microlesions 18
DNA sequencing of *cytochrome b* and *p53* 18
Microsatellites 20
Tc1/Sma1 fingerprinting 20
Subcontract to screen for K-*ras* mutations 20
RESULTS 20
Survival and Abnormality 20
Flow Cytometry 20
Androgenesis 20
Screens for Microlesions 21
Cytochrome *b* 21
Tumor suppressor gene *p53* 21
Microsatellites 22
Tc1/Sma1 fingerprinting 22
Oncogene K-*ras* 22
DISCUSSION 22
Gene Detection Using the Polymerase Chain Reaction 24
LITERATURE CITED 25

LIST OF FIGURES

Page

1. Comparative nucleotide sequences of cytochrome *b* 42
2. Comparative nucleotide sequences for exons 7 - 10 of *p53* 44

LIST OF TABLES

Page

1. Percent of abnormal embryos and percent of embryos that survived to the eye stage from families incubated below the second screen in incubators with oiled and control (unoiled) gravel 36

2. Mean survival from cleavage to streak and from cleavage to eye for haploid androgenetic embryos derived from males oiled at the highest level and males incubated in clean gravel 37

3. Parental haplotypes for nt 486 at the 5' end of codon 162 of cytochrome *b* sequenced with primer H15498. Number of progeny, incubated in oiled and control substrate, with A and G 38

4. Numbers of pink salmon screened and number of nucleotides (nt) screened from families incubated in oiled and unoiled gravel at Little Port Walter, AK, for each of the five *p53* primers used 39

5. Genotypes of fish, originating from the Little Port Walter (LPW) experiment, corresponding to base pair 1,054 of *p53* in rainbow trout 40

6. Genotypes corresponding to base pair 1,054 of *p53* in rainbow trout cDNA 41

LIST OF APPENDICES

Page

A. Intensive Flow Cytometry Analyses do not Detect Macrolesions in Pink Salmon Embryos Following Exposure to Crude Oil 46

B. Analysis of Deleterious Mutations in Pink Salmon using Haploid Androgenesis 62

C. An Initial Evaluation of Molecular Genetic Damage to Oiled Pink Salmon Populations in Prince William Sound 78

D. Use of Microsatellites in Two Genetic Studies of Alaskan Salmon (Oncorhynchus spp.) 85

E. SINE and Transposon Sequences Generate High-Resolution DNA Fingerprints: Screening for Genetic Damage in Pink Salmon (Oncorhynchus gorbuscha) Exposed to Prudhoe Bay Crude Oil 91

F. An Evaluation Of Molecular Genetic Damage To Pink Salmon Embryos Experimentally Exposed To Weathered Prudhoe Bay Crude Oil 109

EXECUTIVE SUMMARY

- Extensive study demonstrated that exposure of pink salmon embryos to *Exxon Valdez* crude oil did not induce genetic damage in the form of macrolesions detectable by flow cytometry, despite indications to the contrary in other species (c.f., Kocan et al. 1996a; Bickham et al. 1998). We had enough statistical power to detect treatment differences as small as the smallest differences detected in similar studies in the literature. Our data do not support the hypothesis that North slope crude oil in incubation substrate, at concentrations up to 5.7-g oil/kg gravel, causes structural chromosome damage in pink salmon embryos.

- Results from pilot studies to use mortality of androgeneic progeny or microsatellite screens to detect genetic damage did not prove useful. In the pilot study, natural male to male variability eroded the power of the androgenesis screen to detect mortality differences between treatments of less than 14%; the microsatellite screen was abandoned upon recommendation of reviewers.

- We developed PCR-based technique to obtain high resolution fluorescent DNA fingerprints and used this approach to test loss or gain of alleles in response to oil exposure. A dye-labeled primer complementary to the *Tc1*-like transposon together with an unlabeled primer for the *SmaI* SINE repeat motif were used to simultaneously amplify between 50 and 80 fragments ranging in size from 75 to 550 bases using PCR. No loss or gain in oil-exposed treatments was detected in our experiments, indicating that overall chromosomal integrity was maintained without significant insertions or deletions.

- DNA sequencing of the sentinel loci cytochrome *b* and *p53* detected no mutations occurred in response to crude oil exposure.

- High-frequency mutations due to oil exposure were detected in the K-*ras* oncogene. PCR and direct DNA sequence analyses were used to identify mutational hotspots within exons 1 and 2 of K-*ras* and 3' primer mismatch analysis was used to determine the frequency of mutations in the 40 offspring of two families of pink salmon which were experimentally exposed to oiled gravel. Mutations were only observed at codons 12, 13, and 61 of K-*ras*, sites which are frequently mutated in animal and human tumors. The frequencies of mutations in oiled embryos at K-*ras* exons 1 and 2 were 68% and 41%, respectively. K-*ras* mutations were not observed in siblings that were exposed to clean gravel or in the parents of the two experimental matings.

Overview: The 9x191A project series was designed to monitor recovery of pink salmon *Oncorhynchus gorbuscha* populations in Prince William Sound that were impacted by the 1989 *Exxon Valdez* oil spill. Here we report results from the component of the study designed to screen for genetic damage in pink salmon subjected to controlled oilings. The potential of crude oil to induce genetic damage was examined from the fall 1992 through 1997.

Embryo mortality was significantly greater in oil-contaminated streams than in non-contaminated reference streams in 1989 and 1990. The differences were observed in all

intertidal areas in 1989, and differences were only observed in the highest intertidal area in 1990. These results were consistent with observations of intertidal oiling from other studies: among oiled streams, all intertidal areas were contaminated in 1989 whereas in 1990 oil remained only in the upper intertidal zone.

The 1991 evaluation demonstrated significant difference in embryo mortality between oil-contaminated and reference streams with differences occurring in both the intertidal and upstream zones. This finding was unexpected, as the presence of oil was dramatically reduced in all areas, and investigators hypothesized that:

(1) the difference in embryo mortality was due to environmental factors that differed uniformly between oiled and non-oiled streams, or
(2) oil-induced damage to the 1989 brood was transmitted multi-generationally within the oiled populations (either through germline genetic-damage or through physiological damage to adults expressed in their progeny).

Both hypotheses were supportable. The environmental-difference hypothesis seemed credible because, in fact, it was environmental factors (wind and currents) that determined the fate of the oil. Such environmental factors might also influence the survivability of salmon embryos incubating intertidally. The genetic-damage hypothesis seemed credible because oil is a known clastogenic substance (breaks chromosomes), and pink salmon have an obligate two-year life cycle. The pink salmon which spawned during the fall of 1991 were from the 1989 brood year, the brood year which incubated in oiled gravel during the fall of 1989 and spring of 1990. Also, a pattern of embryo mortality similar to, but not as extreme as 1991, was observed in 1992 and 1993. The 1993 embryos were two generations removed from the oil exposure in 1989.

In 1993 and 1994, controlled incubation experiments were performed to test the hypothesis that the differences in pink salmon embryo mortality observed in recent years were due to naturally occurring environmental differences. Gametes were collected from spawning adults taken from eight oil-contaminated and eight reference streams. The gametes were flown to the Armin F. Koernig (AFK) Hatchery in southwest Prince William Sound where intra-stream crosses were made, and the resulting embryos from each stream were placed in a common incubator. The 1993 pink salmon embryos from oil-contaminated streams showed elevated mortalities when compared to the embryos from reference streams. This finding clearly indicated that the elevated embryo mortalities observed in the field monitoring portion of the study were not due to systematic differences between the incubating environments of oiled and reference streams. The embryo incubation experiment was repeated in 1994, but no significant difference in embryo mortality between oil-contaminated and reference streams was detected. These negative results in 1994 were consistent with results obtained from the field monitoring component of this project in 1994 where no differences were seen.

In 1992 and 1993, we looked for potential chromosome damage by testing individuals of known oiling history for the presence of DNA macrolesions using flow cytometry. Flow cytometry is a technique previously reported to provide a sensitive screen for clastogenic events to other taxa

similarly exposed to petrochemicals and other toxic substances. No evidence of clastogenic damage was detected. We concluded that, if the elevated embryo mortality is the result of oil-induced genetic damage, such damage is not measurable using flow cytometry in our experimental design.

In 1995 we analyzed pink salmon of known oiling history with another flow cytometric analysis. Unlike the previous experiments, we controlled for family and time of sampling and analysis effects which resulted in increased power to detect macrolesion damage. Despite our power to detect differences as small as those reported in the literature for similar studies, we did not detect any differences between the highly oiled and control treatments. We concluded that Prudhoe Bay crude oil does not induce detectable macrolesion damage in pink salmon.

Washington State University (WSU) used an androgenetic (paternal-only inheritance) screen for deleterious mutations in 1995. In this test, sperm from pink salmon of known oiling history returning to the Little Port Walter (LPW) Hatchery operated by National Marine Fisheries Service (NMFS) was used to fertilize enucleated eggs; the death/time curve of the embryos was theorized to accelerate in individuals possessing genetic damage from oil. No differences were detected in overall survival or in mortality rates between haploids derived from males that were incubated as embryos in the an intermediate dose (see Heintz et al. 1995) and males incubated in the unoiled incubators. However, the power of this experiment to detect differences was very low due to variability in survival of embryos derived from different males within the same treatments. Only with at least a 14% difference in the survival of embryos between the two most extreme treatments, at the stage when eye pigment was first visible, would we have had a reasonable (80%) chance of detecting differences statistically.

Evidence from other laboratories presented since the inception of this study in 1992 suggested that genotoxic damage may be better detected through polymerase-chain-reaction (PCR) based assays, especially those capable of detecting single-base-pair mutations. We initially chose to screen the mitochondrial DNA gene cytochrome *b* and the tumor suppressor gene *p53* as potential sentinel loci for detecting differences in mutation rates between embryos incubated in oiled and unoiled gravel. Protocols were optimized in 1995, but no differences were observed in the initial study. Nucleotide sequences were found to be highly conserved, even in the exons of *p53* that are reported to be hypervariable in other species.

Because existing techniques for mutation detection are generally limited to screening specific, preselected genomic targets, in 1996 we developed a novel technique to fingerprint genomes and applied this technique to screen for mutations. This DNA fingerprinting approach has the advantage that no *a priori* selection of target loci is made, yet one can perform high throughput screens for insertions/deletions as small as two base pairs in many loci dispersed throughout the genome. A dye-labeled primer complementary to the *Tc1*-like transposon together with an unlabeled primer for the *SmaI* short interspersed elements (SINE) repeat motif are used to simultaneously amplify between 50 and 70 independent loci ranging in size from 60 to 535 nucleotides (nt). These fluorescently labeled DNAs are separated on a denaturing acrylamide gel and resolved using an automated DNA sequencing instrument. We found the complex pattern of amplified fragments to be a reliable, specific fingerprint for the individual. In a comparison of DNA fingerprints from 195 oiled and 187 control embryos, no specific mutations (loss or gain of

alleles) were observed. These results indicate that no significant insertion/deletion events occurred affecting loci amplified in our screen. One limitation of this technique is that it cannot detect single nucleotide substitutions.

The final experiment in this project series was a 1996-1997 subcontract to Isaac Wirgin, New York University Medical School, for an assessment of possible point mutations in the K-*ras* oncogene. Wirgin's laboratory used PCR analysis to screen for alterations in the K-*ras* oncogene in DNA from pink salmon embryos that were exposed under controlled laboratory conditions to weathered *Exxon Valdez* crude oil. PCR and direct DNA sequence analyses were used to identify mutational hotspots within exons 1 and 2 of K-*ras* and 3' primer mismatch analysis was used to determine the frequency of mutations in the 40 offspring within each of two families of pink salmon which were experimentally exposed to oiled or clean gravel. Mutations were only observed at codons 12, 13, and 61 of K-*ras*, sites which are frequently mutated in animal and human tumors. All mutations resulted in deduced amino acid substitutions. As expected, in all individuals exhibiting mutations, copy number of the normal allele exceeded that of the mutated allele. The frequencies of mutations in oiled embryos at K-*ras* exons 1 and 2 were 68% and 41%, respectively. K-*ras* mutations were not observed in siblings that were exposed to clean gravel or in the parents of the two experimental matings. These results indicate that exposure of pink salmon embryos to weathered *Exxon Valdez* crude oil under controlled laboratory conditions can elicit somatic cell mutations in high frequency at mutational hotspots in genes such as K-*ras*. However, the frequency of these events in oiled natural populations of pink salmon and other vulnerable species in Prince William Sound and the heritability of these mutations within oiled lineages has yet to be evaluated.

During the conduct of this project we successfully adapted existing strategies and developed new strategies for gene detection in salmonids. Ancillary benefits include the development of dozens of new markers valuable for gene mapping and population genetic studies funded by the Trustee Council and other agencies and universities.

INTRODUCTION

Wild salmon play a major role in the Prince William Sound ecosystem while also contributing to the region's commercial fisheries. Migrating salmon fry are an important food source in the spring for various mammals, birds, and fishes. Marine mammals prey on the ocean life stages of Pacific salmon while terrestrial mammals and birds, such as bears, river otters, eagles, and gulls depend on salmon for a large portion of their summer diet. Salmon also provide a pathway for transferring nutrients from marine ecosystems to nearshore and terrestrial ecosystems. In recent years, commercial catches of wild pink salmon *Oncorhynchus gorbuscha* in Prince William Sound have ranged from 10 to 15 million fish.

Up to 75% of spawning pink salmon in Prince William Sound use intertidal areas (Helle et al. 1964). These areas are highly susceptible to contamination from marine oil spills. Rice et al. (1975) and Moles et al. (1987) found that pink salmon embryos and pre-emergent fry were adversely affected by exposure to crude oil and that the affect was most acute in intertidal environments. The March 24, 1989, spill from the *Exxon Valdez* occurred just prior to the spring

migration of salmon fry and contaminated many intertidal spawning areas in central and southwest Prince William Sound.

Embryo mortality was significantly greater in oiled streams in 1989 and 1990 ($P = 0.004$ and $P = 0.023$) with the differences being in all intertidal areas in 1989 and in the highest intertidal area in 1990 (Bue et al. 1996). These results were consistent with the observations of Wolfe et al. (1994) on intertidal oil-contamination: among oiled streams, all intertidal areas were contaminated in 1989 whereas in 1990 visible oil remained only in the upper intertidal zone.

The 1991 evaluation detected a larger difference in embryo mortality between oil-contaminated and reference streams ($P = 0.003$) than had been previously recorded (Bue et al. 1996). This finding was unexpected and raised several questions about the source of the differing mortality, including the possibility that oil-induced damage was transmitted genetically. Petrochemicals (including *Exxon Valdez* crude oil) have been shown to damage chromosomes (Longwell 1977; McBee and Bickham 1988; Kocan et al. 1996a; Bickham et al. 1998). The pink salmon which spawned during the fall of 1991 were from the 1989 brood year, the brood year which incubated in oiled gravel during the fall of 1989 and spring of 1990. A pattern of embryo mortality similar to, but not as extreme as 1991, was observed in 1992 ($P=0.001$) and 1993 ($P=0.023$) (Bue et al. 1996; Bue et al. 1998), suggesting multigenerational effects.

These field findings were interesting, but they did not provide direct evidence that genetic damage caused by exposure to crude oil affected embryo survival. Reasonable alternatives proposed during the Trustee Council review process to explain the persistent mortality differences included: (1) systematic, naturally occurring environmental differences between oiled and reference streams, and (2) crude oil, hypothetically remaining in the sediments through 1991, continued to have toxic effects that manifested as elevated embryo mortality through 1993.

During the summer of 1993 we evaluated the systematic environmental difference hypothesis. Results from replicates of controlled matings clearly showed that embryos from oiled lineages, incubated in identical conditions to those from non-oiled lineages, had the same elevated embryo mortalities as detected in the field observations ($p = 0.012$, Bue et al. 1998). This finding effectively eliminated the possibility that naturally occurring environmental differences could account for the elevated mortality.

The possibility that the mortality difference was due to physiological effects of crude oil remaining in the sediment through 1991 is difficult to assess. Exposure of the 1991 embryos to toxic oil could explain elevated mortality in the 1993 progeny (e.g., see Truscott et al. 1983; Thomas and Budiantara 1995). Evidence exists that toxic effects were manifested in embryos incubated in microenvironments, trapped oil pockets, within some of the previously oiled sites, but no systematic evaluation was made to evaluate the pervasiveness of this phenomenon (see Wiedmer et al. 1996).

The focus of this component of Project 9x191 was to evaluate the genetic damage hypothesis. Some of the polycyclic aromatic hydrocarbons (PAHs) present in crude oil have been implicated as genotoxic carcinogens and mutagens. The association between PAHs and liver tumors is well established in several fish species (Fong et al. 1993; Hendricks et al. 1994; Rotchell et al. 1995),

and progress is being made toward understanding the molecular events linking PAH exposure to carcinogenesis. Increased incidence of DNA adduct formation is correlated with increased exposure to PAHs (Fong et al. 1993; Stein et al. 1994), elevated intracellular concentrations of free radicals are associated with PAH exposure (Vethaak et al. 1990), induction of detoxification enzymes such as cytochrome *P450* has been measured (Stegemann and Kloepper-Sama 1987) and, finally, reports are accumulating documenting site-specific DNA mutations associated with exposure (Fong et al. 1993; Hendricks et al. 1994; Omer et al. 1995). We described field data showing that populations of pink salmon from oiled streams have suffered multigenerational damage, and our aim was to test for a relationship between exposure to *Exxon Valdez* crude oil and mutation. Tests included a flow cytometric examination for chromosome breakage; an androgenesis screen for deleterious recessive mutations; and development of DNA-sequence-based screens for mutations in pink salmon including sequencing of the mitochondrial DNA (mtDNA) locus cytochrome *b* and the nuclear DNA (nDNA) *p53* tumor suppressor gene and the K-*ras* oncogene, microsatellite assays, and development of a novel method of DNA fingerprinting to screen the size of multiple DNA templates with finescale resolving power.

Flow Cytometry

Flow cytometry is known to be a rapid and effective technique for detecting macrolesion clastogenic effects of petrochemicals and other genotoxins (McBee and Bickham 1988; Lamb et al. 1991; Custer et al. 1994). In a comparison of assays designed to detect genotoxic response, Bickham (1990) found flow cytometry to be less costly, less labor intensive, faster, and as sensitive as other cytogenetic techniques. For these reasons we chose flow cytometry to test the germline-damage hypothesis in pink salmon in controlled experiments.

Flow cytometry measures DNA content of individual cells which can be displayed in histograms. Measurements from these histograms used to assess genetic damage (in the form of clastogenesis--chromosome breaks and rearrangements) include: the coefficient of variation (CV = standard deviation/mean) of the G1 peak (Kocan and Powell 1985; Kocan et al. 1985; Bickham et al. 1988; McBee and Bickham 1988; Custer et al. 1994; Fisher et al. 1994; Biradar and Rayburn 1996); the percent of cells in the S-phase (Jenner et al. 1990); the ratio of the number of cells in the G1 peak relative to the G2 peak (G1/G2 ratio; Jenner et al. 1990); and presence of shoulders off the G1 peak that could not be accounted for by sliced nuclei or cells in the G1 peak or S-phase (Bickham et al. 1988; McBee and Bickham 1988; Jenner et al. 1990). Although our previous work demonstrated no evidence of clastogenesis in embryos exposed to crude oil (Miller et al. 1994; Seeb et al. 1995), peer review comments of our 1994 annual report indicated that a reexamination of the flow cytometry approach was warranted in FY 1995 in order to demonstrate that we had the statistical power to detect the magnitudes of differences reported in the literature for similar studies. Without an experimental design with a statistical analysis of the power, it is difficult to determine if the lack of a detectable difference was due to Type II error or to a lack of an effect.

Androgenesis

Haploid androgenesis has been used in fish as a sensitive assay for mutagenic chemicals (Armstrong and Fletcher 1983) and harmful mutations (Streisinger et al. 1981). Mortality in progeny of treated and untreated fish provides a means of directly measuring heritable genetic damage. The advantage of using haploids over diploids in the screening for deleterious mutations in gametes is that both dominant and recessive deleterious mutations are expressed in haploid progeny. Although non-mutated haploid salmonids die before hatch, those individuals with deleterious mutations die earlier (Thorgaard 1994). Therefore, the mortality curves of progeny from treated and untreated fish may provide a sensitive assay for deleterious mutations.

Androgenetic haploids are more feasible to use than are gynogenetic haploids when large numbers of sperm from treated individuals are easier to obtain than are eggs. To produce androgenetic haploids, eggs are irradiated with ionizing radiation to destroy the maternal chromosomes and then fertilized (Parsons and Thorgaard 1984).

Development of Mutation Screens for Microlesions in Pink Salmon

Mitochondrial DNA

We chose to initially emphasize comparative sequence assays on the mitochondrial genome for several reasons. In mammalian species the mitochondrial genome experiences a 25 times greater rate of nucleotide substitution than nDNA (Lynch 1996). Ectothermic vertebrates appear to evolve at a somewhat slower rate, although the ratio of base substitution (mtDNA/nDNA) still shows a tenfold increase in molecular evolution in the mitochondria (Meyer 1993). The fidelity of DNA replication in the mitochondria is also reduced, and mtDNA repair systems are less stringent (Alberts et al. 1994). Lynch (1996) not only confirmed that mtDNA accumulates mutations much faster than nDNA, but also showed that even deleterious mutations become evolutionarily fixed in many species due to the relaxed control over DNA repair and replication.

Additionally, novel mutations in mtDNA are frequently associated with genetic disease (Brockington et al. 1993; Fryer et al. 1994; Goto et al. 1994; Hayashi et al. 1994; reviewed in Brown and Wallace 1994; Torroni and Wallace 1994; Wallace 1994a,b). Many of these mutations are point mutations (e.g., Hammans et al. 1993; Munscher et al. 1993; Sakuta et al. 1993; Hattori et al. 1994; Jun et al. 1994; Juvonen et al. 1994; Merante et al. 1994), mandating DNA sequence analysis for detection (see also Houshmand et al. 1994). Germline transmission of mtDNA lesions has been implicated (Silvestri 1994; Holme et al. 1995; Shoubridge 1995), further indicating mtDNA to be a reasonable starting place for our study. Baker et al. (1996) found that the base-pair substitution rates for the cytochrome *b* gene in voles living near the Chernobyl nuclear reactor were hundreds of times greater than is typically found for vertebrates, further evidence for the lack of stringent DNA-repair in the mitochondria. These data make cytochrome *b* a prime target for somatic mutation studies. The mtDNA cytochrome *b* gene has also been shown to be a diagnostic, mutationally active gene for studies of molecular evolution of salmonids (Shedlock et al. 1992; Palsson and Arnason 1994; Patarnello et al. 1994; Bernatchez and Osinov 1995).

Tumor Suppressor Genes and Oncogenes

Establishing the molecular link between environmental carcinogens and the onset of tumors has been an area of active research. A distinct set of genes is found to mutate following exposure to specific carcinogens. These include tumor suppressor genes and oncogenes. The products of mutated (also called 'activated') oncogenes interact with regulators of cell proliferation, DNA repair systems, and other components of inter- and intracellular signal transduction pathways (reviewed by Van Beneden 1993; Malkin 1994). That site-specific mutations occur in specific genes has been well documented; for example, mutations within certain 'hot-spot' regions of the tumor suppressor gene *p53* are associated with about 50% of all human cancers (Harris 1993). Most mutations are found clustered in exons 5-8 of this gene, allowing efficient screening for DNA sequence alterations. Intense work sequencing this gene from many tumors has now identified more than 5000 cases of nucleotide substitutions (Hollstein et al. 1996). Studies on the *Ha-ras* oncogene show that specific mutational events at distinct codons are associated with different chemical treatments (DiGiovanni et al. 1993). The capacity of many carcinogens, including PAHs, to cause point mutations in hot spot regions makes a comparative sequencing of these regions in crude-oil exposed pink salmon an obvious direction for our efforts.

Microsatellites

An initial assay of microsatellite loci was included in this project because they have high rates of natural mutation and are emerging as a useful class of genetic markers for study of genetic processes in salmonids (Wright and Bentzen 1994). Interestingly, genetic change documented in tumorous tissue in humans includes a loss of microsatellite heterozygosity within *p53* introns (e.g., Hahn et al. 1995).

DNA fingerprinting

Existing techniques for mutation detection are generally limited to screening specific, pre-selected genomic targets. To overcome this limitation, we developed a novel technique to fingerprint genomes and have applied this technique to screen for genotoxic mutations in pink salmon exposed to crude oil. This DNA fingerprinting approach has the advantages that no *a priori* selection of target loci is made, yet one can perform high throughput screens for small-scale rearrangements or insertions/deletions as small as two base pairs in many loci dispersed throughout the genome.

OBJECTIVES

Our objectives were to test the following three hypotheses relating to the presence of genetic damage in pink salmon embryos exposed to *Exxon Valdez* crude oil:

1) Clastogenic damage is more common in pink salmon exposed to crude oil during development than those not exposed.

2) Androgenetic progeny from males exposed to crude oil during development have faster mortality rates than androgenetic progeny from males not exposed.

3) Point mutations at sentinel mDNA sites or tumor supressor genes or oncogenes are more common in pink salmon exposed to crude oil during development than those not exposed.

METHODS

Tissues for assay in this project originated from two sources. First, initial adaptation and development of primers and optimization of protocols were largely done on oiled and control populations originating from experiments conducted by NMFS at Little Port Walter Hatchery during Project 94191B (see Heintz et. al. 1995). Forty six individuals from a population incubated in oiled gravel and 46 individuals from the same population incubated in clean gravel were used. Subsequent development of assays and expansion of the implementation of assays was done on progeny from single-pair matings and oilings done at the ADF&G laboratory in Anchorage.

Controlled Matings and Oilings Done at the ADF&G Laboratory in Anchorage

In September 1995, eggs from 20 females returning to AFK Hatchery in Prince William Sound were removed into reclosable freezer bags, and 5 ml of milt from each of 20 males was placed into 15 ml capped centrifuge tubes. Gametes were placed on wet ice and flown to the ADF&G Genetics Laboratory in Anchorage. Within 10 hours of gamete collection, 20 single-pair matings were performed. From each mating, fertilized eggs were divided in two equal lots; one lot was placed in an incubator with oiled gravel, and the other lot was placed in an incubator with unoiled gravel. The first set of ten matings were deposited into the gravel of 10 oiled and 10 unoiled incubators. A perforated plate was added above the gravel in all incubators, and the second set of 10 matings were placed above this plate resulting in two single-pair matings per incubator separated from each other by a perforated plate.

Incubators for oil exposure were designed so water flowed upwards through a column of gravel, simulating the incubating environment preferred by salmon (see Figure 1 in Appendix A). Incubators were constructed of 60 cm sections of 15 cm diameter polyvinylchloride (PVC) pipe placed on end and sealed with a PVC plate glued to the bottom. Water was admitted through a 1.9 cm diameter hole drilled and tapped into the side of the pipe immediately above the bottom plate. Flow to each incubator was regulated with a valve. A plate of polypropylene with 4 mm diameter holes on 8 mm centers was fixed inside the pipe, providing a false bottom that

suspended gravel in the incubator 3.8 cm above the bottom plate. The polypropylene plate diffused the water flow through the column of gravel. Water exited the incubators 12.5 cm from the top through a 1.9 cm diameter hole drilled and tapped into the side of the incubator. Incubators were filled with 10.8 kg river gravel with a maximum diameter of 5.1 cm. Gravel was washed to remove fine sediment and allowed to dry prior to application of oil.

Gravel was oiled with 5.7 g oil/kg gravel using North Slope crude oil (obtained from the NMFS, Auke Bay Laboratory's supply of *Exxon Valdez* crude oil). Oil was sprayed evenly onto the gravel as it tumbled in a cement mixer. After oil was applied, gravel was spread into a single layer and exposed to sun for four days before being placed in the incubators. After the gravel was placed into the incubators, water was allowed to flow through the gravel for seven days before embryos were introduced into the incubators.

Two more polypropylene plates with the same perforation pattern as the false bottom plate were used to contain the two single-pair matings incubated in each incubator. A fiberglass insect mesh was attached to the top of these plates with thermoplastic cement to prevent eggs or larvae from passing. The first plate was placed above 7 kg of gravel, and the second was placed above all of the gravel after the first set of matings were introduced into the incubators.

Incubation temperatures averaged 5.6°C with a range from 5.0°C to 6.5°C. Flow was maintained between 170 and 230 ml per minute and adjusted three times a week. Prophylactic treatments to control fungus, consisting of 13ppt NaCl, were administered twice a week for one hour. Dissolved oxygen concentrations in incubator effluents were measured during hatching and were all above 90% of saturation.

Between 24 to 37 days post-fertilization, coinciding with neurulation, some embryos from the families incubated above the second screens were sampled for flow cytometric analysis (see Appendix A for methods). Between 55 and 67 days post-fertilization, when eyes showed pigmentation, embryos and unfertilized eggs from all matings (both above and below the second screen) were extracted from the incubators, dropped 30 cm onto a hard surface, counted, and classified as dead, abnormal, or normal. Opaque embryos/eggs were classified as dead, embryos with irregularly small eyes were classified as abnormal, and those with standard-sized eyes as normal. For matings below the second screens, survival and abnormality proportions were calculated, and paired T-test performed to test for differences between treatments. Survival and abnormality proportions for matings above the second screen were not tested because sampling for flow cytometry occurred in these family-treatments when the embryos were at a fragile stage. For screens for genetic effects, embryos were dissected into 95% reagent-grade alcohol one week after the survival and abnormality data were taken.

Flow Cytometry

The methods, results, and discussion of the flow cytometry component of this project are described in Appendix A.

Androgenesis

Methods used by Washington State University (WSU) for this component are described in Appendix B. Washington State University was to rely upon rearing of males of known oiling history to sexual maturity in marine net pens at Little Port Walter Hatchery. Natural catastrophe resulted in the loss of all of those fish (see Project 9x191-B series annual reports to the Trustee Council). However, Little Port Walter Hatchery staff insightfully released a few thousand fish from the 1993 brood, surplus from some doses, into the marine environment. A few males from medium- to low-dose exposures returned in 1995 and were used in this study (Appendix B).

To clarify the WSU experimental design, it is important to note that each "experiment" consisted of matings done on a different egg batch. Egg batches were suspected to vary in quality due to shipping and handling varaibles inherent in transporting samples from the remote facility, and the data were consequently re-examined using a randomized complete block analysis, where egg batches formed blocks. Because different numbers of males from each treatment were used in each experiment, egg batch and treatment effects were confounded. For example, if a greater proportion of oiled males relative to control males fertilized a high quality egg batch and the opposite proportions fertilized a low quality egg batch, the survival of the embryos from the oil-treated males will be overestimated. We invoked Proc GLM (SAS 1988) to untangle treatment and block effects. In addition we used an arc-sine-square-root transformation in order to stabilize the variances of the residuals between treatments. Finally, we performed a power analysis to determine the limits of our ability to detect differences. This analysis provides a basis for determining how sensitive our experimental design was in detecting differences thereby allowing interpretation of our inability to reject the null hypothesis.

Polymerase-Chain-Reaction Based Screens for Microlesions

No primers were available that had been developed in pink salmon. We collected candidate primer sequences from studies of other species, adapted protocols to optimize priming in pink salmon, and when necessary developed primer subsets to break larger sites into templates no longer than 400nt, a size easily manageable in our sequence analyses. Initial development of primers was generally done on the oiled and non-oiled populations available from Little Port Walter Hatchery (the only oil-exposed individuals available at the time). Subsequent focus was on the single-pair matings described above.

DNA sequencing of cytochrome *b* and *p53*

Immediately prior to DNA analyses, embryos were dissected from the yolk and placed into alcohol in separate 1.5mL vials. After dissection, embryos were coded so that treatment was hidden during analysis. DNA extractions from embryonic tissues were accomplished using the Gentra Systems (Minneapolis, MN) Puregene Kit following the manufacturer's instructions. DNA was isolated from adult frozen tissues using standard methods (Sambrook et al. 1989).

The cytochrome *b* region of mtDNA was PCR amplified using a Perkin-Elmer (P-E; Foster City, CA) 9600 thermalcycler. Primers used were:

LGL-765 5' GAA AAA CCA YCG TTG TWA TTC AAC T 3' (Cronin et al. 1993)
H15498 5' GGA ATA AGT TAT CTG GGT CTC 3' (Kocher et al, 1989).

The total size of the cytochrome *b* region amplified between the LGL-765 and H15498 primers was 795nt. In this study, we focused on the 400nt sequence detectable from the H15498 end. However we could generate sequences 400nt long from both primers, overlapping in the center. We developed a mid-cytochrome *b* primer at the 3' end of the sequence generated by the primer LGL-765. This allowed us to better characterize H15498-derived template by sequencing in both directions.

A 1066 nt region of the tumor-suppressor gene *p53* was also amplified on a P-E 9600 thermalcycler. The region included exons seven through ten, and primers used were:

p53-7F1 5' CAG GTG GGA TCA GAG TGT ACC 3'
p53-10R1 5' AGC GTC GGC AAC AGG CAC CAA CTC 3'.

The *P53*-7F1 primer was provided by Dr. Linda Park, NMFS, Seattle Washington. The *p53*-10R1 primer was developed by selecting a conserved region found through comparison of exon 10 sequence available from Dr. Park for chinook salmon and the rainbow trout sequence (de Fromentel et al. 1992). Additional primers were developed to subdivide the 1066nt *p53* template by selecting optimal 20mers about 400nt towards the center of the template from the two ends.

Cytochrome *b* PCR conditions were as follows: 75 ng of DNA were amplified in 50ul containing 1.25mM dNTPs, 1.5mM $MgCl_2$, 1X PCR buffer (P-E), 60uM each primer, and 2.5U Amplitaq (P-E). An initial denaturation at 97°C for one min was followed by 40 cycles of 15 s at 95°C, one min at 54°C, and two min at 72°C. This was followed by a final extension of five min at 72°C. The *p53* template was amplified as for cytochrome *b* but with 20uM each primer, and 40 cycles of 12 s at 94°C, 30 s at 60°C, and 70 s at 72°C.

Cytochrome *b* and *p53* amplified products were gel-purified using 0.8% Sea Plaque GTG agarose FMC (Rockland, ME). Excised bands were purified using the QIAQuick Gel Extraction Kit (Qiagen; Chatsworth, CA) following the manufacturer's instructions. Purified products were run through a Microcon 100 filter (Amicon; Beverly, MA) to remove residual salts. Cycle-sequencing was done in 20ul reactions using 6ul of purified DNA, 8ul Applied Biosystems Inc (ABI; Foster City, CA) Dye-Terminator Ready Reaction Mix (P-E), 32 pmol primer, 2.8ul dH_2O, following the manufacturer's instructions.

Centri-Sep columns (Princeton Separations; Adelphia, NJ) were used to remove unincorporated dye-terminators following the manufacturer's instructions. Samples were dried and resuspended in deionized formamide: 25mM EDTA with 5mg/ml Dextran Blue (5:1). The samples were denatured at 95°C for 2 min, placed immediately on ice, loaded on a 4.65% acrylamide, 7M urea gel, and run for 3.5 h on the ABI 377 automated DNA sequencer. Sequence data were analyzed with Sequence Navigator software (ABI).

Cytochrome *b* polymorphisms were confirmed through forward and reverse manual sequencing by an external laboratory (Appendix C).

Microsatellites

The methods, results, and discussion of the microsatellite component of this project are described in Appendix D.

Tc1/SmaI fingerprinting

The methods, results, and discussion of the *Tc1/SmaI* fingerprinting component of this project are described in Appendix E.

Subcontract to screen for K-*ras* mutations

The methods, results, and discussion of the NYU subcontract are described in Appendix F.

RESULTS

Survival and Abnormality

Significantly lower survival was observed in the oiled incubators (mean = 17%) than in the control incubators (mean = 23%, $P = 0.012$, Table 1). In addition, significantly higher abnormality rates were observed in the oiled incubators (mean = 16%) than the control incubators (mean = 0%, $P = 0.016$, Table 1). We did not include data for incubator #2 in these tests because DNA fingerprint results showed that embryos from the family incubated above the screen dropped into the family incubating below (Appendix E); therefore other data collected from this incubator could be corrupt.

Flow Cytometry

No flow cytometric evidence of clastogenic damage was detected despite the high power of the experiment to detect such damage (Appendix A).

Androgenesis

Washington State University used an androgenetic (paternal-only inheritance) screen for deleterious mutations in 1995 (Appendix B). They detected no differences in survival to streak or to eye between progeny of males incubated in oiled and progeny of males incubated in control gravel.

We reanalyzed their data blocking by "experiment" (shipment-to-shipment egg quality variation) and using an arc-sine transformation to normalize the data. We did this reanalysis because

different numbers of males from each treatment were used in each experiment. Analyzing the data as a block without factoring out shipment-to-shipment variability in egg quality could lead to biases and inflated experimental errors.

In our reanalysis of their data in the haploid androgen experiment, we also did not detect differences in the survival of progeny from the highest dose and control treatments (Table 2). The power analysis for the cleavage-to-eye test indicated that there would have had to have been at least a 14% difference in the survival of embryos between the highest dose and the control treatments at the stage when eye pigment was first visible before we would have had a reasonable (80%) chance of detecting it statistically with a = 0.05.

Screens for Microlesions

Cytochrome *b*

The mid-cytochrome *b* primer developed in this study (5'-TAT CGC CCG GGG ACT TTA TTA TGG-3') allowed us to sequence the 400nt template originating a H15498 in both directions. This proved valuable because we encountered difficulty in scoring nucleotide 486 in codon 162. False heteroplasmy was observed in the progeny of female 2A, but this was clarified by the confirming sequence in the opposite direction. No mutations were observed in the sequence of the 629 individuals for the 400nt sequenced (Table 3).

Unexpectedly little variation was found in the DNA sequence of cytochrome *b* in the preliminary screen of 20 parents for this 400nt (Table 3). Nucleotide 486 in codon 162 was an A in all individuals except female 2A which was a homoplasmic G. Six-hundred and twenty-nine progeny were sequenced from the 20 pairs of families using primer H15498 (Table 3).

Tumor suppressor gene *p53*

The 1066nt template was sequenced in three subcomponents (Table 4) after development and optimization of the three internal primers:

p53-8R1 5' CCG ACC CAG GCG CTG CCC 3'

p53-9R1 5' GAG GGG CAG GCA GGG AGG CC 3'

p53-9F1 5' GGC CTC CCT GCC TGC CCC TC 3'

We found no evidence of mutations in response to oil and little variation in our initial screen of the *p53* gene in individuals originating from the oiled population from LPW Hatchery (Table 5). The pilot screen of a few progeny from the single-pair matings also revealed no evidence of mutation in response to oil (Table 6); but interestingly, the base pair corresponding to nt 1,054 of *p53* in rainbow trout copy DNA (cDNA; de Fromentel et al. 1992) was polymorphic in pink salmon (Figure 2) obtained both from LPW Hatchery and from AFK Hatchery.

Microsatellites

See Appendix D.

Tc1/SmaI fingerprinting

See Appendix E.

Oncogene K-*ras*

The frequencies of mutations in oiled embryos at K-*ras* exons 1 and 2 were 68% and 41%, respectively. K-*ras* mutations were not observed in siblings that were exposed to clean gravel or in the parents of the two experimental matings. Mutations were only observed at codons 12, 13, and 61 of K-*ras*, sites which are frequently mutated in animal and human tumors. All mutations resulted in deduced amino acid substitutions. As expected, in all individuals exhibiting mutations, copy number of the normal allele exceeded that of the mutated allele. See Appendix F.

DISCUSSION

Our objectives were to test the hypotheses that 1) macrolesions are more common in pink salmon exposed to crude oil during development than those not exposed, 2) androgenetic progeny from males exposed to crude oil during development have faster mortality rates than androgenetic progeny from males not exposed, and 3) microlesions are more common in pink salmon embryos exposed to crude oil during development than those not exposed.

We conducted this study because pink salmon embryos that incubated in oil-contaminated spawning areas in Prince William Sound suffered elevated mortalities. Bue et al. (1998) found increased pink salmon embryo mortalities in 1989, 1990, 1991, 1992, and 1993. We believe that the elevated mortalities observed in 1989 and 1990 were due to direct exposure to oil; elevated mortalities observed in 1991, 1992, and 1993 may have been due to genetic damage sustained in the parental lines during embryonic development in 1989 and 1990 that was inherited and affected subsequent generations.

The pink salmon adults that spawned during the fall of 1991 incubated as embryos in oil-contaminated streams during winter of 1989-1990, the first winter after the spill. Likewise, the pink salmon adults that spawned during the fall of 1992 incubated as embryos in oiled stream gravel during the fall of 1990 and spring of 1991. Bue et al. (1998) found significantly elevated embryo mortalities in oil-contaminated streams during the fall of 1989 and 1990, and the surviving embryos may have sustained sublethal genetic damages which were manifested in the form of reduced embryo survival in 1991, 1992 and 1993. Chromosome damage was observed in other taxa exposed to petrochemicals (Longwell 1977; McBee and Bickham 1988; Kocan et al. 1996a); documentation of germline damage is yet to be reported.

One alternative to the genetic damage hypothesis is that observed differences in embryo mortality are due to systematic environmental differences. The embryo mortality study is based on observational data, and as such, we were unable to randomize stream oiling to account for environmental differences between streams. We attempted to address this concern in our original experimental design by selecting unoiled or reference streams in close proximity to oil-contaminated streams; however, there is a definite oiling pattern in southwest Prince William Sound whereby streams on points which faced northeastward were heavily oiled. Likewise, streams which faced west and southwest were most likely not oiled. Finally, oiled streams may have had residual oil not found in the unoiled streams.

However, we interpret the results from the controlled incubation experiments conducted at AFK Hatchery to suggest that the basis for the differences in embryo mortality observed in the field was not solely due to variation in the incubation environment. The gametes used in this study had never been in contact with a stream; although, the adults which produced them had incubated in the natal streams. Still, these data do not prove that the differences were caused by oil contamination. Some have suggested that the streams which were oiled also historically had lower embryo survival.

The results of our matings and experimental oilings in this study parallel those of LPW Hatchery (Project 94191B, Heintz et al. 1995) where elevated mortality and abnormality rates were observed in embryos incubated in effluent from oiled gravel. Our experimental design included incubation of embryos in contact with the oiled substrate, increasing the chance that we would observe the effects of PAH toxicity (c.f., Kocan et al. 1996b).

Our flow cytometry analysis of pink salmon embryos and larvae exposed to crude oil failed to detect evidence of DNA macrolesions (Appendix A). This lack of a correlation was found despite field and laboratory evidence suggesting the presence of genetic damage and the high power of the experimental design. If genetic damage was responsible for the differential mortality observed in the field and laboratory studies, then flow cytometry may not be the test of choice for detecting the genetic effects of PAHs, especially in salmonids. Subtle clastogenic effects may be masked by the sometimes large intraspecific variation of DNA content present within a taxon (Lockwood and Bickham 1991; Sherwood and Patton 1982; Gold and Price 1985). In addition, DNA macrolesions may be particularly difficult to detect in salmonids, given their tetraploid ancestry and large intraspecific DNA content variation (Allendorf and Thorgaard 1984; Johnson et al. 1987). Alternatively, it appears likely *Exxon Valdez* crude oil simply may not induce macrolesions in the DNA of pink salmon embryos.

We believe that the haploid androgenesis test for deleterious mutations may be a powerful tool for studying genetic damage even though it had limited value as applied in this study. This component relied on the production of mature males of known oiling history. Attempts to rear such males in saltwater net pens for the two years necessary have been fraught with natural disaster (see Project 9x191-B series annual reports).

Little Port Walter staff had the insight to release a few thousands of experimental animals in 1993 which provided the few individuals available for this study. But numbers were small, and only individuals from the medium to low exposures were recovered in 1995. Additional

drawbacks to these experiments included the limitation encountered in applying the radiation treatment rapidly to large numbers of salmon eggs and variability encountered due to handling of the different shipments of gametes from LPW Hatchery to WSU. The power analysis for the cleavage-to-eye test indicated that there would have had to have been at least a 14% difference in the survival of embryos at the eyed stage between the highest dose and the control treatments before we would have had a reasonable (80%) chance of detecting it statistically with alpha = 0.05. Differences of this magnitude *or less* could account for the differences in embryo mortality observed in the field. WSU proposes to further refine the protocols and replication strategy necessary to detect the presence of differences among treatments, but we currently have no plans to repeat this experiment given the daunting challenge of rearing pink salmon to maturity in net pens.

Gene Detection Using the Polymerase Chain Reaction

The focus has switched, in studies of genetic damage in fish, from cytogenetic and flow cytometric assays toward more PCR-based assays since this project was initiated in 1992 (see reviews in Bailey et al. 1996; Roy et al. 1999). We surveyed the utility of an array of PCR-based assays including DNA sequencing, microsatellite analysis, and DNA fingerprinting.

We initially screened nine microsatellite loci developed in other salmonid species for interspecific priming using pink salmon DNA. We found that four of these, Oneμ1, Oneμ11, μSat60, and μSat73, worked well. We screened the oiled and non-oiled populations from the LPW experiments at these four loci for alteration in allelic expression in heart, liver, kidney and spleen. No loss or gain of alleles was detected. Additional development suggests that microsatellites will have substantial application in gene detection studies in salmonids (Olsen et al. 1996), but based upon our initial results we do not plan to pursue this approach in the immediate future.

Because existing techniques for mutation detection are generally limited to screening specific, preselected genomic targets, we developed a novel PCR-based technique to fingerprint genomes and applied this technique to screen for mutations. This DNA fingerprinting approach has the advantages that no *a priori* selection of target loci is made, and one can perform high throughput screens for insertions/deletions as small as two base pairs in many loci dispersed throughout the genome. A dye-labeled primer complementary to the *Tc1*-like transposon together with an unlabeled primer for the *SmaI* SINE repeat motif are used to simultaneously amplify between 50 and 70 loci ranging in size from 60 to 535nt. These fluorescently labeled DNAs are separated on a denaturing acrylamide gel and resolved using an automated DNA sequencing instrument. We found the complex pattern of amplified fragments to be a reliable, specific fingerprint for the individual. In a comparison of DNA fingerprints from 195 oiled and 187 control embryos, no specific mutations (loss or gain of alleles) were observed. These results indicate that no significant insertion/deletion events occurred affecting loci amplified in our screen. However, one limitation of this technique is that it cannot detect single nucleotide substitutions (see for example Holme et al. 1995).

The most promising approach for detection of PAH-induced genetic damage may prove to be DNA sequence analysis capable of detecting single nucleotide substitutions (c.f., Houshmand et al. 1994). Studies increasingly demonstrate the role of somatic point mutations in genetic damage and genetic disease and cancer (e.g., Merante et al. 1994; Santorelli et al. 1994), and the germline implications of some mutations are profound (Yeung et al. 1994; Silvestri et al. 1994; Shoubridge 1995).

We observed lower levels of naturally occurring variation in cytochrome *b* and *p53* than were expected based upon observations of other taxa. For *p53*, only one nucleotide was found polymorphic in all of the individuals sequenced for our 1066nt template. The polymorphism, corresponding to nt 1,054 of *p53* in rainbow trout cDNA (de Fromentel et al. 1992), was observed at low frequency in both the oiled and non oiled fish from LPW Hatchery. Interestingly, this same base was found to be polymorphic in the six parents initially screened from AFK Hatchery. The assay of a few embryos from three pairs of the full-sib-single-pair matings made with these parents demonstrated no difference between oiled and non-oiled treatments.

It is important to note that the automated assay that we use provides a conservative observation of mutation because mutations in response to genotoxic challenge would be expected to yield mosaic genotypes. Mosaicism often goes undetected on both manual and automated sequencing gels, and the software in our instrument would not score a mutation unless it was present in 50 per cent or more of the cells from which the DNA was extracted and amplified. However, many of the point mutations implicated in response to genotoxic challenge and genetic disease are site-specific (see for example Fong et al. 1993; Bowles et al. 1994; Loechler 1996; Sugio et al. 1996). The approach used in this study (Appendix F) was to sequence to identify candidate sites and then design more sensitive mismatch analysis to re-examine treatment individuals for the presence of the mutations at that site.

LITERATURE CITED

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1994. Molecular Biology of the Cell. Garland Pub. New York.

Allendorf, F. W., and G. H. Thorgaard. 1984. Tetraploidy and the evolution of salmonid fishes. *In* B.J. Turner (ed) Evolutionary genetics of fishes. Plenum Press, New York, pp 1-53.

Armstrong, J. B. and W. S. Fletcher. 1983. Assessment of mutagenic damage following ethyl methanesulonate mutagenesis in the axolotl (*Ambystoma mexicanum*) J. Exp. Zool. 226:333-340.

Bailey, G. S., D. E. Williams, and J. D. Hendricks. 1996. Fish models for environmental carcinogenesis--the rainbow trout. Environmental Health Perspectives 104:5-21.

Baker, R. J., R. A. Van Den Bussche, A. J. Wright, L. E. Wiggens, M. J. Hamliton, E. P. Reat, M. H. Smith, M. D. Lomakin, and R. K. Chesser. 1996. High levels of genetic change in rodents of Chernobyl. Nature 380:707-708.

Berger, R, M. Flexor, M. Le Coniat, and C. Larsen. 1996. Presence of three recurrent chromosomal rearrangements, t(2;3)(p12;q37), del(8)(q24), and t(14;18) in an acute lymphoblastic leukemia. Cancer Gen. Cytogen. 86:76-79.

Bernatchez, L., and A. Osinov. 1995. Genetic diversity of trout (genus Salmo) from its most eastern native range based on mitochondrial DNA and nuclear gene variation. Mol. Ecol. 4:285-297.

Bickham, J. W., B. G. Hanks, M. J. Smolen, T. Lamb, and J. W. Gibbons. 1988. Flow cytometric analysis of the effects of low-level radiation exposure on natural populations of slider turtles (*Pseudemys scripta*). Arch. Environ. Contam. Toxicol. 20:837-841.

Bickham, J. W. and M. J. Smolen. 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ. Health Perspect. 102:25-28.

Bickham, J. W. 1990. Flow cytometry as a technique to monitor the effects of environmental genotoxins on wildlife populations. *In* pp 97-108 Sandhu S. S., W. R. Lower, F. J. de Serres, W. A. Suk, R. R. Tice (eds) In Situ Evaluations of Biological Hazards of Environmental Pollutants. Plenum Press, New York.

Bickham, J. W., J. A. Mazet, J. Blake, M. J., Smolen, L. Yigong, and B. E. Ballachey. 1998 Flow cytometric determination of genotoxic effects of exposure to petroleum in mink and sea otters. Ecotoxicology 7:191-199.

Biradar, D. P. and A. L. Rayburn. 1995. Chromosomal damage induced by herbicide contamination at concentrations observed in public water supplies. Journal of Environmental Quality 24(6):1222-1225.

Bowles, N., D. Bonnet, F. Mulhauser, and P. F. Spahr. 1994. Site-directed mutagenesis of the P2 region of the rous sarcoma virus GAG gene--effects on GAG polyprotein processing. Virology 203:20-28.

Brockington, M., M. G. Sweeney, S. R. Hammans, J. A. Morganhughes, and A. E. Harding. 1993. A tandem duplication in the D-loop of human mitochondrial DNA is associated with deletions in mitochondrial myopathies. Nature Genetics 4:67-71.

Brown, M. D. and D. C. Wallace. 1994. Molecular basis of mitochondrial DNA disease. [Review]. Journal of Bioenergetics and Biomembranes 26:273-289.

Bue, B. G., S. Sharr, S. D. Moffitt, and A. K. Craig. 1996. Effects of the *Exxon Vladez* oil spill on pink salmon embryos and preemergent fry. American Fisheries Symposium 18:619-627.

Bue, B. G., S. Sharr, and J. E. Seeb. 1998. Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the *Exxon Valdez* oil spill. Transactions of the American Fisheries Society, 127, 35-43.

Burke, T., O. Hanotte, M. W. Bruford, and E. Cairns. 1991. Multilocus and single locus minisatellite analysis in population biological studies. *In*: DNA Fingerprinting: Approaches and Applications. Burke T., G. Dolf, A. J. Jeffreys and R. Wolf [eds.] Birkhauser Verlag. Basel, Switzerland.

Cronin, M. A., W. J. Spearman, R. L. Wilmot, J. C. Patton, and J. W Bickham. 1993. Mitochondrial DNA variation in chinook salmon (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Canadian Journal of Fisheries and Aquatic Sciences, 50(4), 708-715.

Custer, T. W., J. W. Bickham, T. B. Lyne, T. Lewis, L. A. Ruedas, C. M. Custer, and M. J. Melancon. 1994. Flow cytometry for monitoring contaminant exposure in black-crowned night-herons. Archives of Environmental Contamination & Toxicology 27(2):176-179.

de Fromentel, C. C., F. Pakdel, A. Chapus, C. Baney, P. May, and T. Soussi. 1992. Rainbow trout *p53*:cDNA cloning and biochemical characterization. Gene 112: 241-245.

Denko, N. C., A. J. Giaccia, J. R. Stringer, and P. J. Stambrook. 1994. The human *Ha-ras* oncogene induces genomic instability in murine fibroblasts within one cell cycle. Proc. Natl. Acad. Sci. U.S.A. 91:5124-5128.

DiGiovanni, J., L. Beltran, A. Rupp, R. G. Harvey, and R. D. Gill. 1993. Further analysis of *c-Ha-ras* mutations in papillomas initiated by several polycyclic aromatic hydrocarbons and papillomas from uninitiated, promoter-treated skin in SENCAR mice. Mol. Carcinogen. 8:272-279.

Dubrova, Y. E., D. N. Nesterov, N. G. Krouchinsky, V. A. Ostapinko, R. Neumann, D. L. Neil, and A. J. Jeffreys. 1996. Human minisatellite mutation rate after the Chernobyl accident. Nature 380:683-686.

Fisher, S. K., C. E. Dallas, C. Jagoe, M. H. Smith, I. L. Brisbin, and R. K. Chesser. 1994. Sources of error associated with sample collection and preparation of nucleated blood cells for flow cytometric analyses. Cell Biology and Toxicology 10(3):145-153.

Fong, A. T., R. H. Dashwood, R. Cheng, C. Mathews, B. Ford, J. D. Hendricks, and G. S. Bailey. 1993. Carcinogenicity, metabolism and *Ki-ras* proto-oncogene activation by 7,12-dimethylbenz[a]anthracene in rainbow trout embryos. Carcinogenesis 14:629-635.

Fryer, A., R. Appleton, M. G. Sweeney, L. Rosenbloom, and A. E. Harding. 1994. Mitochondrial DNA 8993(NARP) mutation presenting with a heterogeneous phenotype including cerebral palsy. Archives of Disease in Childhood 71:419-422.

Gandini, D., S. Moretti, A. Latorraca, C. De Angeli, F. Lanza, A. Cueno, G. Castoldi, and L. del Senno. 1996. p53 exon 5 mutations in two cases of leukimic mantle cell lymphoma. Cancer Gen. Cytogen. 86:120-123.

Geiger, H. J., B. G. Bue, S. Sharr, A. C. Wertheimer, and T. M. Willette. 1996. A life history approach to estimating damage to Prince William Sound pink salmon from the *Exxon Valdez* oil spill. *In* Rice, S. D., R. B. Spies, D. A. Wolfe, and B. A. Wright [eds.] Exxon Valdez Oil Spill Symposium Proceedings. Am. Fish. Soc. Symp. 18:487-498.

Gold, J. R. and J. H. Price. 1985. Genome size variation among North American minnows (Cyprinidae). I. Distribution of the variation in five species. Heredity 54:297-305.

Goto, Y. I., K. Tsugane, Y. Tanabe, I. Nonaka, and S. Horai. 1994. A new point mutation at nucleotide pair 3291 of the mitochondrial tRNA(LEU(UUR)) gene in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Biochemical & Biophysical Research Communications 202:1624-1630.

Hahn, M., S. E. Matzen, J. Serth, and A. Pingoud. 1995. Semiautomated quantitative detection of loss of heterozygosity in tumor suppressor gene *p*53. Biotechniques 18(6):1040-1047.

Hammans, S. R., M. G. Sweeney, M. Brockington, G. G. Lennox, N. F. Lawton, C. R. Kennedy, J. A. Morganhughes, and A. E. Harding. 1993. The mitochondrial DNA transfer RNA(LYS) A-]G(8344) mutation and the syndrome of myoclonic epilepsy with ragged red fibres (MERRF) - relationship of clinical phenotype to proportion of mutant mitochondrial DNA. Brain 116:617-632.

Harris, C. C. 1993. *p53*: At the crossroads of molecular carcinogenesis and risk assessment. Science 262:1980-1981.

Hattori, Y., Y. Goto, R. Sakuta, I. Nonaka, Y. Mizuno, and S. Horai. 1994. Point mutations in the mitochondrial TRNA genes - sequence analysis of chronic progressive external ophthalmoplegia (CPEO). Journal of the Neurological Sciences 125:50-55.

Hayashi, J. I., S. Ohta, Y. Kagawa, D. Takai, S. Miyabayashi, K. Tada, H. Fukushima, K. Inui, S. Okada, Y. Goto, and I. Nonaka. 1994. Functional and morphological abnormalities of mitochondria in human cells containing mitochondrial DNA with pathogenic point mutations in TRNA genes. Journal of Biological Chemistry 269:19060-19066.

Heintz, R. A., S. D. Rice, and J. W. Short. 1995. Injury to pink salmon eggs and preemergent fry incubated in oiled gravel (laboratory study), *Exxon Valdez* Oil Spill Restoration

Project Annual Report (Restoration Project 94191-2), NOAA, NMFS, Auke Bay Laboratory, Juneau, Alaska.

Helle, J. H., R. S. Williamson, and J. E. Bailey. 1964. Intertidal ecology and life history of pink salmon at Olsen Creek, Prince William Sound, Alaska. U.S. Fish and Wildlife Service, Special Scientific Report--Fisheries No. 483. Washington, D.C.

Hendricks, J. D., R. S. Cheng, D. W. Shelton, C. B. Pereira, and G. S. Bailey. 1994. Dose-dependent carcinogenicity and frequent *Ki-ras* proto-oncogene activation by dietary N-nitrosodiethymamine in rainbow trout. Fundamental and Applied Toxicology 23:53-62.

Hollstein, M., B. Shomer, T. Greenblat, T. Soussi, E. Hovig, R. Montesano, and C. C. Harris. 1996. Somatic point mutations in the *p53* gene of human tumors and cell lines: updated compilation. Nuc. Acids Res. 24:141-146.

Holme, E., M. H. Tulinius, N. G. Larsson, and A. Oldfors. 1995. Inheritance and expression of mitochondrial DNA point mutations. Biochimica et Biophysica Acta - Molecular Basis of Disease 1271:249-252.

Houshmand, M., N. G. Larsson, E. Holme, A. Oldfors, M. H. Tulinius, and O. Andersen. 1994. Automatic sequencing of mitochondrial TRNA genes in patients with mitochondrial encephalomyopathy. Biochimica et Biophysica Acta - Molecular Basis of Disease 1226:49-55.

Jenner, N. K., G. K. Ostrander, T. J. Kavanagh, J. C. Livesey, M. W. Shen, S. C. Kim, and E. H. Holms. 1990. A flow cytometric comparison of DNA content and glutathione levels in hepatocytes of English sole (*Parophyrs vetulus*) from areas of differing water quality. Archives of Environmental Contamination and Toxicology 19:807-815.

Johnson, O. W., F. M. Utter, and P. S. Rabinovitch. 1987. Interspecies differences in salmonid cellular DNA identified by flow cytometry. Copeia 4:1001-1009.

Jun, A. S., M. D. Brown, and D. C. Wallace. 1994. A mitochondrial DNA mutation at nucleotide pair 14459 of the NADH dehydrogenase subunit 6 gene associated with maternally inherited Leber hereditary optic neuropathy and dystonia. Proceedings of the National Academy of Sciences of the United States of America 91:6206-6210.

Juvonen, V., K. Huoponen, A. C. Syvanen, E. Nikoskelainen, and M. L. Savontaus. 1994. Quantification of point mutations associated with Leber hereditary optic neuroretinopathy by solid-phase minisequencing. Human Genetics 93:16-20.

Kocan, R. M. and D. B. Powell. 1985. Anaphase aberrations: an in vitro test for assessing the genotoxicity of individual chemicals and complex mixtures. *In* pp75-85 Walters M.D., S.S. Sandhu, J. Lewta, L. Claxton, G. Strauss, and S. Nesnow (eds). Short-term Bioassays in the Analysis of Complex Environmental Mixtures IV. Plenum Publishing Corp.

Kocan, R. M., D. P. Powell, and V. M. Liguori. 1985. Relationship between visible and heritable chromosome damage in trout cells and embryos. Marine Environmental Research 17:320-323.

Kocan, R. M., J. E. Hose, E. D. Brown, and T. T. Baker. 1996a. Pacific herring (*Clupea pallasi*) embryo sensitivity to Prudhoe Bay petroleum hydrocarbons: laboratory evaluation and in situ exposure at oiled and unoiled sites in Prince William Sound. Canadian Journal of Fisheries and Aquatic Sciences 53:2366-2375.

Kocan, R. M., M. B. Matta, and S. M. Salazar. 1996b. Toxicity of weathered coal tar for shortnose sturgeon (*Acipenser brevirostrum*) embryos and larvae. Archives of Environmental Contamination and Toxicology 31(2):161-165.

Kocher, T. D., W.K Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca, and A.C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals. Proc. Natl. Acad. Sci. USA 86:6186-6200.

Lamb, T., J. W. Bickham, J. Whitfield Gibbons, M. J. Smolen, and S. McDowell. 1991. Genetic damage in a population of slider turtles (*Trachemys scripta*) inhabiting a radioactive reservoir. Arch. Environ. Contam. Toxicol. 20:138-142.

Lockwood, S. F., and J. W. Bickham. 1991. Genetic stock assessment of spawning Arctic cisco (*Coregonus autumnalis*) populations by flow cytometric determination of DNA content. Cytometry 12:260-267.

Loechler, E. L. 1996. The role of adduct site-specific mutagenesis in understanding how carcinogen-DNA adducts cause mutations. Carcinogenesis 17(5):895-902.

Longwell, A. C. 1977. A genetic look at fish eggs and oil. Oceanus 20(4):46-58.

Lydall, D. and T. Weinert. 1995. Yeast checkpoint genes in DNA damage processing: Implications for repair and arrest. Science 270:1488-1491.

Lynch, M. 1996. Mutation accumulation in transfer RNAs: molecular evidence for muller's ratchet in mitochondrial genomes. Mol. Biol. Evol. 13:209-220.

Malkin, D. 1994. Germline *p53* mutations and heritable cancer. Ann. Rev. Genet. 28:443-465.

Mauchly, J. W. 1940. Significance test for sphericity of a normal n-variate distribution. Annals of Mathematical Statistics Vol. 11.

Mayr, B., G. Schaffner, R. Kurzbauer, M. Reifinger, and K. Schellander. 1995. Sequence of an exon of tumor suppressor *p53* gene--a comparative study in domestic animals--mutation in a feline solid mammary carcinoma. British Veterinary Journal 151:325-329.

McBee, K. and J. W. Bickham. 1988. Petrochemical-related DNA damage in wild rodents detected by flow cytometry. Bull. Environ. Contam. Toxicol. 40:343-349.

Merante, F., I. Tein, L. Benson, and B. H. Robinson. 1994. Maternally inherited hypertrophic cardiomyopathy due to a novel T-to-C transition at nucleotide 9997 in the mitochondrial tRNA (glycine) gene. American Journal of Human Genetics 55:437-446.

Miller, G. D., J. E. Seeb, B. G. Bue, and S. Sharr. 1994. Saltwater exposure at fertilization induces ploidy alterations, including mosaicism, in salmonids. Can. J. Fish. Aquat. Sci. 51:42-49.

Moles, A., M. M. Babcock, and S. D. Rice. 1987. Effects of oil exposure on pink salmon, *O. gorbuscha*, alevins in a simulated intertidal environment. Marine Environmental Research 21:49-58.

Munscher, C., T. Rieger, J. Mullerhocker, and B. Kadenbach. 1993. The point mutation of mitochondrial DNA characteristic for MERRF disease in found also in healthy people of different ages. FEBS Letters 317:27-30.

Neter, J., W. Wasserman, and M. H. Kutner. 1990. Applied Linear Statistical Models - Third Edition. Irwin. Homewood, Illinois.

Nielsen, P.S., N. de Pater, H. Okkles, and H. Autrup. 1996. Environmental air pollution and DNA adducts in Copenhagen bus drivers- effect of GSTM1 and NAT2 genotypes on adduct levels. Carcinogenesis 17(5):283-288.

Olsen, J. B., J. K. Wenburg, and P. Bentzen. 1996. Semi-automated multilocus genotyping of Pacific salmon (Oncorhynchus spp.) using microsatellites. Mol. Mar. Biol. Biotechnol. 5:259-272.

Orner, G.A., C. Mathews, J. D. Hendricks, H. M. Carpenter, and G. S. Bailey. 1995. Dehydropiandrosterone is a complete hepatocarcinogen and potent tumor promoter in the absence of peroxisome proliferation in rainbow trout. Carcinogenesis 16:2893-2898.

Page, J. E., H. L. Ross, C. A. H. Bigger, and A. Dipple. 1996. Mutagenic specificities and adduct formation for 7-bromomethylbenz[a/it>]anthracenes. Carcinogenesis 17(2):283-288.

Palsson, S., and E. Arnason. 1994. Sequence variation for cytochrome *b* genes of three salmonid species from Iceland. Aquaculture 128:29-39.

Parsons, J. E., and G. H. Thorgaard. 1984. Induced androgenesis in rainbow trout. J. Exp. Zool. 231:407-412.

Patarnello, T., L. Bargelloni, F. Caldara, and L. Colombo. 1994. Cytochrome *b* and 16S rRNA sequence variation in the *Salmo trutta* (Salmonidae, Teleosti) species complex. Mol. Phylog. Evol. 3:69-74.

Pirtle, R. B. and M. L. McCurdy. 1977. Prince William Sound general districts 1976 pink and chum salmon aerial and ground escapement surveys and consequent brood year egg deposition and preemergent fry index programs. Alaska Department of Fish and Game, Division of Commercial Fisheries, Technical Data Report 9. Juneau, AK.

Rice, S. D., D. A. Moles, and J. W. Short. 1975. The effect of Prudhoe Bay crude oil on survival and growth of eggs, alevins, and fry of pink salmon, *Oncorhynchus gorbuscha*. *In*: pp 503-507 Proceedings of the 1975 Conference on Prevention and Control of Oil Pollution, San Francisco, CA. American Petroleum Institute. Washington, D.C.

Rotchell, J. M., R. M. Stagg, and J.A. Craft. 1995. Chemically-induced genetic damage in fish: isolation and characterization of the dab (*Limanda limanda*) ras gene. IAR Poll. Bull. 457-459.

Roy, N.K., J. Stabile, J.E. Seeb, C. Habicht, and I. Wirgin. 1999. High frequency of K-*ras* mutations observed in pink salmon embryos experimentally exposed to *Exxon Valdez* crude oil. Environ. Toxicology and Chemistry 18(7):77-84.

SAS Institute Inc. 1988. SAS/STAT™ User's Guide, Release 6.03 Edition. SAS Institute Inc. Cary, N.C.

Sakuta, R., Y. Goto, I. Nonaka, and S. Horai. 1993. An A-to-G transition at nucleotide pair 11084 in the ND4 gene may be an mtDNA polymorphism. Am. J. Hum. Genet. 53:964-965.

Sagartz, J. E., W. L. Bodley, R. M. Gamblin, C. G. Couto, L. A. Tierney, and C. C. Capen. 1996. *p53* tumor suppressor protein overexpression in osteogenic dogs. Veterinary Pathology 33:213-221.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. New York.

Santorelli, F. M., S. Shanske, A. Macaya, D. C. Devivo, and S. Dimauro. 1993. The mutation at nt 8993 of mtDNA is a common cause of lieghs syndrome. Annals of Neurology 34:827-834.

Seeb, J. E., B. G. Bue, A. K. Craig, C. Habicht, G. D. Miller, and S. Sharr. 1995. Injury to salmon embryos and preemergent fry in Prince William Sound, *Exxon Valdez* oil spill. Restoration Project Final Report (Restoration Project 94191), Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development, Anchorage, Alaska. 106pp.

Sharr, S., B. G. Bue, S. D. Moffitt, and A. K. Craig. 1994a. Injury to salmon eggs and preemergent fry in Prince William Sound - F/S 1. State/Federal Natural Resources Damage Assessment Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, AK.

Sharr, S., J. E. Seeb, B. G. Bue, S. D. Moffitt, A. K. Craig, and G. D. Miller. 1994b. Injury to salmon eggs and preemergent fry in Prince William Sound - R60C. State/Federal Natural Resources Restoration Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, AK.

Sharr, S., J. E. Seeb, B. G. Bue, S. D. Moffitt, A. K. Craig, and G. D. Miller. 1994c. Injury to salmon eggs and preemergent fry in Prince William Sound - R93003. State/Federal Natural Resources Restoration Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, AK.

Shedlock, A. M., J. D. Parker, D. A. Crispin, T. W. Pietsch, and G. C. Burmer. 1992. Evolution of the Salmonid mitochondrial control region. Mol. Phylog. Evol. 1:179-192.

Sherwood, S. W. and J. L. Patton. 1982. Genome evolution in pocket gophers (genus *Thomomys*): II. Variation in cellular DNA content. Chromosoma 85:163-179.

Shoubridge, E. A. 1995. Segregation of mitochondrial DNAs carrying a pathogenic point mutation (TRNA(LEU3243)) in cybrid cells. Biochemical and Biophysical Research Communications 213:189-195.

Silvestri, G., F. M. Santorelli, S. Shanske, C. B. Whitley, L. A. Schimmenti, S. A. Smith, and S. Dimauro. 1994. A new mtDNA mutation in the tRNA(leu(uur)) gene associated with maternally inherited cardiomyopathy. Human Mutation 3:37-43.

Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W.H. Freeman and Company. San Francisco, Ca.

Stegeman, J. J. and P.J. Klopper-Sams. 1987. Cytochrome P-450 isozymes and monooxygenase activity in marine mammals. Environ. Health Perspect. 71:87-95.

Stein, J. E., W. L. Reichart, and U. Varanasi. 1994. Molecular epizootiology: assessment of exposure to genotoxic compounds in teleosts. Environ. Health Perspect. 102:19-23.

Streisinger, G. C., C. Walker, N. Dower, D. Knauber, and F. Singer. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291:293-296.

Sugio, K., A. F. Gazdar, J. Abores-Saavedra, and D. M. Kokkinakis. 1996. High yields of K-ras mutations in intraductal papillary mucinous tumors and invasive adenocarcinomas induced by N-nitroso-(2-hydroxypropyl)amine in the pancreas of female Syrian hamsters. Carcinogenesis 17(2):303-309.

Thomas, P. and L. Budiantara. 1995. Reproductive life stages sensitive to oil and naphthalene in Atlantic croaker. Marine Environmental Research 39:147-150.

Thorgaard, G. H. 1994. Detection of deleterious mutations in pink salmon through haploid androgenesis. Project completion report to Alaska Department of Fish and Game.

Torroni, A., and D. C. Wallace. 1994. Mitochondrial DNA variation in human populations and implications for detection of mitochondrial DNA mutations of pathological significance. Journal of Bioenergetics and Biomembranes 26:261-271.

Truscott, B., J. M. Walsh, M. P. Burton, J. F. Payne, and D. R. Idler. 1983. Effect of acute exposure to crude petroleum on some reproductive hormones in salmon and flounder. Comp. Biochem. Physiol. 75C(1):121-130.

Van Beneden, R. J. 1993. Oncogenes. *In:* Hochachka and Mommsen [eds.] Biochemistry and Molecular Biology of Fishes, vol. 2. Elsevier Science Pub. pp 113-136.

Vethaak, D. and T. A. Rheinallt. 1990. A review and evaluation of the use of fish diseases in the monitoring of marine pollution in the North Sea. ICES CM E:11.

Wallace, D. C. 1994a. Mitochondrial DNA mutation is diseases of energy metabolism. Journal of Bioenergetics and Biomembranes 26:241-250.

Wallace, D. C. 1994b. Mitochondrial DNA sequence variation in human evolution and disease. Proceedings of the National Academy of Sciences of the United States of America 91:8739-8746.

Wanschura, S., G. Belge, G. Stenman, P. Kools, P. D. Cin, E. Shoenmakers, C. Huysmans, H. Van den Berghe, S. Bartnitzke, W. J. M. Van de Ven, and J. Bullerdiek. 1996. Mapping of the translocation breakpoints of primary pleomorphic adenomas and lipomas within a common region of chromosome 12. Cancer Gen. Cytogen. 86:39-45.

Wiedmer, M., M. J. Fink, J. J. Stegeman, R. Smolowitz, G. D. Marty, and D. E. Hinton. 1996. Cytochrome P-450 *induction and histopathology in preemergent pink salmon* from oiled spawning sites in Prince William Sound. *In* Rice, S. D., R. B. Spies, D. A. Wolfe, and B. A. Wright [eds.] Exxon Valdez Oil Spill Symposium Proceedings. Am. Fish. Soc. Symp. 18:509-517.

Williams, B. O., L. Remington, D. M. Albert, S. Mukai, R. T. Bronson, and T. Jacks. 1994. Cooperative tumorigenic effects of germline mutations in *rb* and *p53*. Nature Genetics 7:480-484.

Wolfe, D. A., M. J. Hameedi, J. A. Galt, G. Watabayashi, J. Short, C. Oclaire, S. Rice, J. Michel, J. R. Payne, J. Braddock, S. Hanna, and D. Sale. 1994. The fate of the oil spilled from the *Exxon-Valdez*. Environmental Science and Technology 28:A 560-A 568.

Wright, J. M. and P. Bentzen. 1994. Microsatellites: genetics markers of the future. Reviews in Fish Biology and Fisheries 4:384-388.

Yeung, R. S., G. H. Xiao, F. Jin, W. C. Lee, J. R. Testa, and A. G. Knudson. 1994. Predisposition to renal carcinoma in the eker rat is determined by germ-line mutation of the tuberous sclerosis *2* (*tsc2*) gene. Proceedings of the National Academy of Sciences of the United States of America 91:11413-11416.

Zardoya, R., A. Garrido-Pertierra, and J. Bautista. 1995. The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. J. Mol. Evol. 41:942-951.

Table 1. Percent of abnormal embryos and percent of embryos that survived to the eye stage from families incubated below the second screen in incubators with oiled and control (unoiled) gravel. We did not include data for the incubator #2 set in these tests due to contamination in the oiled incubator of embryos seeded above the screen.

Family #	Survival (%)			Abnormality (%)		
	Oil	Control		Oil	Control	
1	0.6	1.2		0.0	0.0	
3	19.1	23.7		19.4	0.0	
4	11.3	13.1		11.0	0.0	
5	25.1	27.1		12.3	0.0	
7	36.6	38.8		22.7	0.0	
8	11.3	19.4		1.1	0.0	
9	14.0	32.4		53.0	1.2	
10	19.3	28.4		10.0	0.5	
11	16.1	23.3		13.6	0.0	
Mean	17.0	23.0	$P = 0.012$	15.9	0.2	$P = 0.016$

Table 2. Mean survival from cleavage to streak and from cleavage to eye for haploid androgenetic embryos derived from males oiled at the highest level and males incubated in clean gravel. *P* values resulting from testing the hypothesis that control survival is higher than oiled survival are presented. These means and analyses are the product of a reanalysis of the data in Appendix B but blocked by experiment. The cleavage-to-eye analysis was performed with data transformed with an arc-sine-squared transformation to stabilize the variance of the residuals between treatments - reported means are back-transformed values. No differences were observed between the two treatments.

Variable	Treatment		*P* value
	Control	High	
Cleavage-to-streak	19.5%	20.7%	0.705
Cleavage-to-eye	2.83%	1.92%	0.148

Table 3. Parental haplotypes for nt 486 at the 5' end of codon 162 of cytochrome *b* sequenced with primer H15498. Number of progeny, incubated in oiled and control substrate, with A and G in that position are given. Within mating, "A" designates a family incubated above the gravel and "B" indicates families incubated within the gravel. No variation was detected at the other 399 nucleotides in the template sequenced with primer H15498.

Mating	Parental Haplotypes		Treatment	Number of Progeny with Each Haplotype	
	Dam	Sire		A	G
2A	G	A	Oil	0	55
			Control	0	85
2B	A	A	Oil	48	0
			Control	60	0
3B	A	A	Oil	23	0
			Control	24	0
4B	A	A	Oil	25	0
			Control	23	0
5B	A	A	Oil	26	0
			Control	28	0
7B	A	A	Oil	29	0
			Control	32	0
8B	A	A	Oil	17	0
			Control	21	0
9B	A	A	Oil	26	0
			Control	23	0
10B	A	A	Oil	21	0
			Control	19	0
11B	A	A	Oil	23	0
			Control	22	0

Table 4. Numbers of pink salmon screened and number of nucleotides (nt) screened from families incubated in oiled and unoiled gravel at Little Port Walter, AK, for each of the five *p53* primers used.

Primers	Nt screened	Treatment	
		Oiled	Unoiled
7F1	380	32	29
8R1	390	18	10
9R1	429	6	18
9F1	405	11	10
10R1	275	22	10

Table 5. Genotypes of fish, originating from the Little Port Walter (LPW) experiment, corresponding to base pair 1,054 of *p53* in rainbow trout cDNA (de Fromentel et al. 1992). No sequencing data was collected from the parents of the LPW crosses because these progeny were the result of many unpaired matings.

Treatment	Genotype		
	GG	CG	CC
Oil	2	4	5

Table 6. Genotypes corresponding to base pair 1,054 of *p53* in rainbow trout cDNA (de Fromentel et al. 1992). Included are parents and number of their progeny, incubated in oiled and control substrate from single-pair matings. No deviation from Hardy-Weinberg expected frequencies were observed within either oiled or control embryos in the single-pair matings ($P > 0.17$ for all tests).

Cross	Parental genotypes		Treatment	Number of progeny with each genotype		
	Dam	Sire		GG	CG	CC
3B	GG	GG	Oil	9	0	0
			Control	10	0	0
4B	GG	CG	Oil	1	1	0
			Control	6	2	0
11B	CC	CG	Oil	0	4	2
			Control	0	8	4

Figure 1. Comparative nucleotide sequences of cytochrome *b* in rainbow trout (Zardoya et al. 1995) and in pink salmon with the "G" allele and pink salmon with "A" allele at codon 162. Nucleotide bases for codon 162 are in large and bold font. Sequence starts at the 5' end and proceeds toward the 3' end of cytochrome *b*. Periods represent unchanged bases from rainbow trout to pink salmon.

```
Rainbow Trout  ATGGCCAACC TCCGAAAAAC CCACCCTCTC CTAAAAATCG
CTAATGACGC ACTAGTCGAC CTCCCAGCAC
Pink Salmon G  .......... .......... ...T...... ..........
.......... .......... ..........
Pink Salmon A  .......... .......... ...T...... ..........
.......... .......... ..........

Rainbow Trout  CTTCTAATAT CTCAGTCTGG TGAAACTTTG GCTCACTACT
AGGCCTATGT TTAGCTACCC AAATTCTTAC
Pink Salmon G  .A.....C.. .........A .......... .......C..
....T....C C....C.... ..........
Pink Salmon A  .A.....C.. .........A .......... .......C..
....T....C C....C.... ..........

Rainbow Trout  CGGGCTCTTC CTAGCCATGC ACTATACCTC CGACATTTCA
ACAGCTTTCT CCTCTGTTTG CCACATCTGC
Pink Salmon G  ......A... .......... ....C..T.. ..........
........T. .......C.. ..........
Pink Salmon A  ......A... .......... ....C..T.. ..........
........T. .......C.. ..........

Rainbow Trout  CGAGATGTTA GTTACGGCTG ACTCATTCGA AACATCCATG
CCAACGGAGC ATCTTTCTTT TTTATCTGTA
Pink Salmon G  ........C. .C........ ...A.....T ..........
.T........ .......... .....T....
Pink Salmon A  ........C. .C........ ...A.....T ..........
.T........ .......... .....T....

Rainbow Trout  TTTATATACA TATCGCCCGA GGACTTTACT ACGGCTCGTA
CCTCTACAAA GAAACCTGGA ATATCGGAGT
Pink Salmon G  .......G.. .........G ........T. .T..A..A..
...A...... .......... G.........
Pink Salmon A  .......G.. .........G ........T. .T..A..A..
...A...... .......... G.........

Rainbow Trout  TGTACTTTTA CTTCTCACTA TAATAACTGC CTTTGTAGGC
TACGTCCTCC CGTGAGGACA AATATCATTC
Pink Salmon G  .......... .......... .......... A..C......
.......... .......... ...G..C...
```

Pink Salmon A .... A..C......
....G..C...

Rainbow Trout TGAGGGGCCA CTGTAATTAC AAACCTCCTC TCAGCTGTAC
CATACGTAGG AGGCGCCCTA GTA**CAA**TGAA
Pink Salmon G ....T..T ..C.....T.
.C..T..G.. C......... .....**G**..G.
Pink Salmon A ....T..T ..C.....T.
.C..T..G.. C......... .....**A**..G.

Rainbow Trout TTTGAGGGGG CTTCTCCGTT GACAACGCCA CTCTAACACG
ATTTTTCGCC TTTCACTTCC TATTCCCCTT
Pink Salmon G ....C.. A......... ..T....... .C........
....T. ....T..
Pink Salmon A ....C.. A......... ..T....... .C........
....T. ....T..

Rainbow Trout CGTCATTGCA GCCGCTACGG TCCTTCACCT TCTGTTCCTT
CATGAAACAG GATCTAATAA CCCTGCAGGG
Pink Salmon G ......C... ..T.....A. .... C..A......
.....G.... ....C.. ...G......
Pink Salmon A ......C... ..T.....A. .... C..A......
.....G.... ....C.. ...G......

Rainbow Trout ATTAACTCTG ATGCTGATAA AATCTCATTC CACCCTTACT
TCTCATACAA AGATCTCCTA GGATTCGTAG
Pink Salmon G ....C. ....C..... ......G..T ....
....G..... ...C.....C ..G.......
Pink Salmon A ....C. ....C..... ......G..T ....
....G..... ...C.....C ..G.......

Rainbow Trout CCATACTCCT AGGCCTAACA TCCTTAGCTC TTTTTGCACC
AAATCTCCTA
Pink Salmon G ....T.. T..T...... ..A.....C. .A........
...CT..T..
Pink Salmon A ....T.. T..T...... ..A.....C. .A........
...CT..T..

Figure 2. Comparative nucleotide sequences for exons 7 - 10 of *p53* in cDNA (exons only) of rainbow trout (de Fromentel et al. 1992) and DNA (exons and introns) of pink salmon. All intron bases are missing for rainbow trout because only copy DNA data is published. Sequence starts at the 5' end and proceeds toward the 3' end of *p53*. Periods represent unchanged bases from rainbow trout to pink salmon and dashes represent positions in the rainbow trout sequence where additional bases are found in pink salmon. Variation of bases in bold were screened among pink salmon exposed to oiled or unoiled gravel from three families. The base pair corresponding to nt 1,054 of *p53* in rainbow trout cDNA (de Fromentel et al. 1992) was polymorphic in pink salmon and is coded as "S" (G or C) in the sequence. No other variation was detected.

```
Rainbow Trout (cDNA) GATCTACAAC TTCATGTGCA ATAGATCCTG TATGGGAGGG
ATGAACCGGA GACCCATCCT CACCATCATC
Pink Salmon          •••••••••• ••••••••T• •C••C••••• ••••••••••
••••••••C• •••••••••• ••••••••••
Rainbow Trout (cDNA) ACCCTGGAGA CACAAGAG
Pink Salmon          ••T••••••• ••••G•••TG AGTGTCATCT AATAATCCAT
TTTAGACTGT TGCTCCATTG ATTGTCTTTG
Rainbow Trout (cDNA)
Pink Salmon          ATTTGTCTTC TCTTTTTCTT CATTTTACTA ATTAATATAA
TAATAATATA TGCCATTTAG CAGACGCTTT
Rainbow Trout (cDNA)
Pink Salmon          TATCCAAAGC GACTTACAGT CATGTGTGCA TACATTCTAC
GTATGGGTGG TCCCGGGAAT CGAACCCACT
Rainbow Trout (cDNA)
Pink Salmon          ACCCTGGCGT TACAAGCGCC ATGCTCTACC AACTGAGCTA
CAGAAGGACC ACAATTAGGA AAAGCATCAT
Rainbow Trout (cDNA)
Pink Salmon          TTGACTGAAT TAAAATAGAA TTGACCCCAA CCCTGGTCCT
GGGTTGACCC CCAACTAATG TCTTGTCATA
Rainbow Trout (cDNA)
GGCAGCTC CTGGGTCGGC GCTCCTTTGA Pink Salmon          GTATGACAGT
GGTGGGAACT TCCTCTCCCC GTTCTTGCAG GG••G••••• ••••••••••
••••••••••
Rainbow Trout (cDNA) GGTGCGTGTG TGTGCCTGTC CTGGTCGAGA CAGGAAGACA
GAGGAGATCA ACCTGAAGAA GCAGCAGGAG Pink Salmon      ••••••••••
•••••••••• •••••••••• •••••G•••• •••••••••• •••••G••T•
•••••••••C
Rainbow Trout (cDNA) ACAACCCTGG AGACCAAGAC CAAGCCTGCC CAGGGAATCA
AACGT
Pink Salmon          •••••••••A ••••••••C• ••••-••••• ••••••TC••
•••••GTAAT TGATCCTTGA CAATCTTAAA
Rainbow Trout (cDNA)
Pink Salmon          CCCGATGGTC TTGGGGTTGG GATTAACATA AAAATGCCAA
AAAGAAAGAT GTCAAGATCT TTGGACAATT
Rainbow Trout (cDNA)
```

Pink Salmon GCACTTTAAA GTTGGATGAT GNCTGATAAA GGTTGACATT
GACCTATGAC CCTGAGTGTG AGCAGTTCCT
Rainbow Trout (cDNA) GCTATGAAG
GAGGCCTCCC TGCCTGCCCC TCAGCCTGGG Pink Salmon CATCGTGTGT
GTCATGTTTG TGTTCTTCTA A••••••••• •••••••••• ••••••••••
•G•••••**A**••

Rainbow Trout (cDNA) GCCAGTAAGA AG--ACCAAG TC----CTCC CCTGCTGTGA
GTGACGATGA GATCTACACT CTTCAG Pink Salmon
•••••••••• ••**ATTCATC**• ••**ACTC**•••• •••••••••• ••••••••••
•••••••••• ••••••**GTAA**
Rainbow Trout (cDNA)
Pink Salmon **CTGCTCAGAG AACAGACCAA TGGAAGTTTT TCTCGTCCCC**
TTCAGAGATG CCTAGTCACT AGTCTGCTGT
Rainbow Trout (cDNA)
AT TCGAGGGAAG GAAAAATATG Pink Salmon **TGGCTGTTTG**
TGTATACTGT GTGTAACTGG GCTCATCTGC CCATCCAG•• ••••••••••
••••••••••
Rainbow Trout (cDNA) AGATGCTGAA GAAGTTCAAT GACAGTCTTG AACTGAGTGA
GTTGGTGCCT GTTGCCGACG C
Pink Salmon •••••••••• •••••••••• •••••••••• ••••••**S**•••
•••••••••• •••••••••• •

Appendix A. Intensive flow cytometry analyses do not detect clastogenic damage in pink salmon embryos following crude oil exposure.

Christopher Habicht, David Evans, and James E. Seeb

Alaska Department of Fish and Game
333 Raspberry Road
Anchorage, Alaska, USA 99518
Fax: (907) 267-2442

Summary

Field data and evidence from experimental matings suggest that germline genetic damage occurred in pink salmon *(Oncorhynchus gorbuscha)* embryos inhabiting streams affected by the 1989 *Exxon Valdez* oil spill. Flow cytometry has proven to be a rapid screen for detection of structural chromosome damage in similar studies of the effects of genotoxins in the environment. However, flow cytometry analysis of pink salmon embryos and larvae exposed in the laboratory to *Exxon Valdez* crude oil failed to detect clastogenic effects in our experiment. We had enough statistical power to detect treatment differences as small as the smallest differences detected in similar studies in the literature. These data do not support the hypothesis that North slope crude oil in incubation substrate, at concentrations up to 5.7-g oil/kg gravel causes structural chromosome damage in pink salmon embryos.

Key words
flow cytometry, structural chromsome damage, DNA, pink salmon embryos, crude oil

Introduction

Studies conducted after the *Exxon Valdez* oil spill, which occurred in Prince William Sound (PWS), Alaska, in March 1989, detected elevated mortalities in pink salmon (*Oncorhynchus gorbuscha*) embryos incubating in streams contaminated by oil. The mortalities persisted two generations after physical evidence of oil contamination in the region had all but disappeared (Bue et al. 1996). In a controlled mating experiment designed to assess the role of the environment in these field findings, when incubated under identical conditions, pink salmon embryos produced from gametes from lineages originating from oiled streams had significantly higher mortalities than those from unoiled reference streams (Bue et al. 1998). These observations led to the hypothesis that the mortality may be caused by germline genetic damage.

The clastogenic effect of crude oil and other, more potent, genotoxins was demonstrated by cytogenic techniques that evaluate the frequency of large chromosomal aberrations (Longwell 1977; Al-Sabti 1985; Kocan and Powell 1985; Daniels and Means 1989). While the utility of cytogenetic techniques for demonstrating the existence of DNA abnormalities such as large deletions and additions for small numbers of individuals with high proportions of chromosomal aberrations is clear, these analyses do not lend themselves to the assessment of genetic damage in large numbers of individuals where large numbers of cells within each individual need to be

screened to identify clastogenetic effects in subpopulations of these cells because they are time consuming and labor intensive.

More recently, flow cytometry was shown to be a rapid and effective technique for detecting clastogenic effects of petrochemicals and other genotoxins (see citations in Table 3). In a comparison of assays designed to detect genotoxicity, Bickham (1990), found flow cytometry to be less costly, less labor intensive, faster, and as sensitive as other cytogenetic techniques. Lamb et al. (1995) found that tissues with high cellular turn-over rates were most sensitive to detection of chromosome damage through flow cytometry. For these reasons we chose flow cytometry of rapidly developing embryos and fry to test the germline-damage hypothesis in pink salmon in controlled experiments.

Flow cytometry measures DNA content of individual cells and are generally displayed in histograms. Chrosmosome breaks (clastogenic damage) are detectable through flow cytometry because the daughter cell lines from cells that have undergone chromosome breakage have slightly more or slightly less DNA content than cell lines from undamaged cells and because cell division might be slower for damaged cells. The coefficient of variation (CV = standard deviation/mean) of the G1 peak can be a good measure of damage and has been used extensively (Kocan and Powell 1985; Kocan et al. 1985; Bickham et al. 1988; McBee and Bickham 1988; George et al. 1991; Lamb et al. 1991; Custer et al. 1994; Fisher et al. 1994; Biradar and Rayburn 1995; Easton et al. 1997; Lowcock et al. 1997; Lingenfelser et al. 1997; Dallas et al. 1998; Taets et al. 1998; Bickham et al. 1998). Other flow cytometric variables used to measure clastogenic damage under the hypothesis that damaged cells take longer to divide which results in higher proportions of cells going though division includes the percent of cells in the S-phase (Jenner et al. 1990; Lingenfelser et al. 1997; Dallas et al. 1998) and the ratio of the number of cells in the G1 peak relative to the G2 peak (G1/G2 ratio; Jenner et al. 1990; Lingenfelser et al. 1997; Dallas 1998). Finally the presence of shoulders off the G1 peak that can not be accounted for by sliced nuclei or cells in the G1 peak or S-phase are also used as indicators of genetic damage with the hypothesis that daughter cell lines with extra DNA are more likely to survive and replicate than daughter cell lines with missing DNA (Bickham et al. 1988; McBee and Bickham 1988; Jenner et al. 1990).

We used flow cytometry in a controlled experiment at the Alaska Department of Fish and Game (ADF&G) Genetics Laboratory in the fall of 1995 to test for clastogenic effects of crude oil exposure during incubation to pink salmon embryos and larvae. This experiment was designed to detect small differences in chromosome damage frequency between embryos incubated in the effluent, above oiled and unoiled substrate by controlling for effects associated with family, date of sampling and analysis (Fisher et al. 1994), and tissue mixtures (Tiersch and Wachtel 1993). We held embryos in substrate effluent until analysis thereby increasing the probability of detecting DNA breakage (Kocan et al. 1985). We tested the effect of incubation substrate on CV of the G1 peak, on percentage of cells in the S-phase, on G1/G2 ratio, and on shoulders off the G1 peak.

No significant differences in structural chromosome damage rates were detected between oiled and reference embryos even though there was sufficient statistical power to detect differences as small as the smallest detected differences published in the literature for the same kinds of studies. We conclude that oil exposure during incubation does not induce clastogenic effects in pink salmon. We suggest that future assessments of heritable genetic damage in pink salmon resulting from oil exposure focus instead on mutational alterations of DNA using more

sensitive approaches that are capable of detecting frameshifts, point mutations, base-pair substitution, and small deletions and additions.

Materials and Methods

Oil-Exposure and Tissue Sampling

To simulate the incubating environment preferred by salmon, incubators used to expose embryos to oil were designed so water flowed up through a column of gravel modified from the design of Marty et al. (1997b). Briefly, incubators were constructed of 60-cm sections of 15-cm-diameter polyvinylchloride (PVC) pipe sealed with a PVC plate glued to one end which became the bottom. Water was admitted near the bottom and was regulated with a valve. A plate provided a false bottom that held gravel in the incubator above the inlet hole. Water exited the incubators through a hole near the top of the incubator. We used a polypropylene plate with 4-mm-diameter holes as the false bottom rather than an aluminum plate as was used by Marty et al. (1997b).

River gravel with maximum diameter of 5.1 was tumbled and rinsed in a cement mixer to remove fines and dried prior to oiling and loading into incubators. Treated gravel was oiled with 5.7-g oil/kg gravel using North Slope crude oil (obtained from the National Marine Fisheries Service, Auke Bay Laboratory's supply of *Exxon Valdez* crude oil). Oil was sprayed evenly onto the gravel as it tumbled in a cement mixer. After oil was applied, gravel was laid out in one layer and exposed to sun for 4 d before being placed in the incubators. Each of 20 incubators was filled with 10.8 kg of either oiled or unoiled gravel. Another polypropylene plate with the same perforation pattern as the false bottom plate was inserted above the gravel before the fertilized eggs used for the flow cytometry analysis were placed into the incubators. To the top of this plate, fiberglass insect mesh was attached with thermoplastic cement. This plate prevented embryos from falling into the gravel, thereby allowing easy access to embryos for sampling while minimizing mortality of unsampled embryos. Water was allowed to run through the gravel for 7 d before embryos were introduced into the incubators.

In September 1995, eggs from 20 females returning to Armin F. Koernig Hatchery in PWS were removed into separate one-gallon reclosable freezer bags, and 5 ml of milt from each of 20 males was placed into separate 15-ml capped centrifuge tubes. Gametes were placed on wet ice and flown to the ADF&G Genetics Laboratory in Anchorage. Within 10 h of gamete collection, 20 single-pair matings were performed. For each mating, fertilized eggs were divided into two groups; half were placed in an incubator with oiled gravel, and half were placed in an incubator with unoiled gravel. The first 10 matings were placed into the 20 incubators without the top plate in place. These matings provided the mortality and abnormality data. The top plate was then inserted and the second set of ten matings were placed into the incubators above this plate. These matings provided the samples analyzed by flow cytometry and were not used to assess mortality and abnormality rates because sampling eggs for flow cytometry likely induced some mortality.

Incubation temperature averaged 5.6°C and ranged from 5.0 to 6.5°C. Flow was maintained between 170 and 230 ml/min and was adjusted three times a week. A prophylactic treatment to control fungus, consisting of 13‰ NaCl, was administered twice a week for 1 h. Dissolved oxygen concentrations in incubator effluents were measured during hatching and were all above 90% of saturation.

Embryos for flow cytometry were sampled from incubators twice, once between days 24 and 37 post-fertilization, which coincided with neurulation, and once between days 55 and 67 post-fertilization, when eyes began to show pigmentation. Sampling and flow cytometry analyses were blocked by family and dates of sampling and analysis. Dissection of whole embryos alternated between treatments within families, and care was taken to avoid contamination between samples. After dissection, embryos were coded and randomized so that treatment was unknown during flow cytometry analysis.

Mortality and abnormality rates from matings incubated below the top plate were measured between 55 and 67 days post-fertilization, when eyes showed pigmentation. Embryos and unfertilized eggs were extracted from the incubators, dropped 30 cm onto a hard surface (which ruptures the chorion in dead eggs resulting in opaque eggs but does not damage live eggs), counted, and classified as dead, abnormal, or normal. Opaque embryos/eggs were classified as dead, embryos with irregularly small eyes were classified as abnormal, and those with standard-sized eyes as normal.

Hydrocarbon levels in tissues from embryos incubated in similarly oiled incubators averaged 246 mg of polyaromatic hydrocarbons per 1 g of tissue (see Marty et al. 1997a for more detailed analysis).

Tissue Preparation and Flow Cytometry

Whole embryos at the neurulation and eyed stages were dissected from the egg by removing the chorion. The yolk sac was then removed using forceps, and the embryos were gently blotted to remove residual yolk material. Immediately after dissection, individual embryos were minced with two scalpels in 0.5 ml of nuclear isolation medium (NIM; modified from Lee et al. 1984: 0.154 M NaCl, 10 mM Tris, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% Noniodet P-40, 106 mM $MgSO_4$ and 1mg /100ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride)) for approximately 30 s to obtain a cellular suspension. The suspension was placed into 1.5 ml microcentrifuge tubes containing 0.5 to 1 ml of NIM (depending on sample volume) to stain nuclei. Samples were vortexed for 3 s, allowed to incubate at 2-3°C for 15 min, vortexed again for 3 s, and filtered through a 40 μm Nitex nylon filter to remove debris and clumped cells. Stained nuclear suspensions were refrigerated overnight (2-3°C) for flow cytometry analysis the following day. Immediately prior to analysis samples were filtered through a 40 μm Nitex nylon filter to remove any residual clumps of nuclei, and diluted with NIM up to a 1:1 ratio depending on cell suspension concentration.

Samples were analyzed using a Partec PAS II flow cytometer with optical filters for DAPI excitation. Acq-cyte data acquisition and Multicycle DNA analysis software (Phoenix Flow Systems Inc., San Diego, CA) were used to generate histograms of DNA content following the methods of Lamb et al. (1991) with a debris curve added to account for sliced nuclei, and for fitting the G1, G2, and S-phase curves. Multicycle uses Gaussian distributions to fit G1 and G2 curves and a polynomial fitted by least squares to model the S-phase curve. Multicycle also calculated the G1 CV; percent of cells in S, G1, and G2 phases; and the G1/G2 ratio.

DAPI-stained and stabilized nuclei from trout erythrocytes (Riese Enterprises, Inc., San Jose, CA) were used as external standards to calibrate the flow cytometer prior to each sampling session and after every sixth sample. The instrument gain controls were used to adjust the standard peak to channel 55 for trout erythrocytes. A distilled water blank was run between samples to remove residual material from the preceding sample. The CV range of the DAPI-

stained standard was routinely 1.8-3.0. Samples were analyzed at flow rates of 50-100 nuclei/s with a total of 19,000 to 21,000 nuclei collected per sample.

Data Analysis

Survival and abnormality proportions were calculated, and paired T-tests performed to test for differences between treatments.

Flow cytometry data were normalized using a natural log transformation. The experiment was analyzed as a randomized complete-block design with subsampling for each of the neurulation and eyed stages of embryo development. There were two treatments (oil and control) and 9 blocks (based on family and date of sampling and analysis) which were treated as random effects. An average of 8.7 embryos was analyzed per treatment-block combination for the neurulation stage and 11.2 for the eyed stage. The experimental unit was a group of embryos representing one of the halves of a family of embryos. The Proc GLM module of SAS (1990) was used to conduct univariate analyses on the G1 CV, the percentage of cells in the S-phase, and the G1/G2 ratio between oil and control treatments using one-tailed tests. The maximum width of the residual area to the right of the G1 peak, representing cells that could not be accounted for by the fitted G1 or S-phase curves was recorded as a measure of the hyperdiploid shoulder (Jenner et al. 1990) and analyzed in a similar manner. Finally, we derived power curves to determine the smallest detectable differences so that we could make comparisons with other studies that used similar methods.

Results

Significantly lower survival was observed in the oiled incubators (mean = 17%) than in the control incubators (mean = 23%, $P = 0.012$, Table 1). In addition, significantly higher abnormality rates were observed in the oiled incubators (mean = 16%) than the control incubators (mean = 0%, $P = 0.016$, Table 1). We did not include data for incubator #2 in these tests because embryos from the family incubated above the screen dropped into the family incubating below; therefore other data collected from this incubator could be corrupt.

No significant effect of oiling was found on any of the variables examined at either the neurulation stage or the eyed stage. These variables included the CV of the G1 peak, the percentage of cells in the S-phase, the G1/G2 ratio, and the hyperdiploid analysis (Table 2). We did, as expected, find a significant block effect at both stages ($P < 0.0001$). Power analyses indicated that we were 90% confident that we could detect differences in G1 CV as small as 0.14, differences in the percent cells in the S-phase as small as 1.11%, and differences in the G1/G2 ratio as small as 1.30 between oiled and control groups. Had the population differences in any of these variables been as small as any of the smallest differences detected in similar studies (Table 3), we would have had between 72 and 100% chance of detecting them for the G1 CV and G1/G2 variables (Figure 1).

Discussion

Increasing concern about pollution in the environment led to a proliferation of assays designed to assess the genotoxic potential of contaminants (first reviewed in Landolt and Kocan 1983). Many of these traditional cytogenetic assays (e.g., the anaphase aberration test) involve

visual scoring of chromosomes, which can be time consuming, subjective, and of low statistical power because of the small sample sizes often used.

Flow cytometry has become an established method for measuring the physical and chemical characteristics of cells (reviewed in Darzynkiewicz and Crissman 1990). Unlike traditional cytometric assays, flow cytometry quantitatively measures thousands of cells per minute making it a powerful tool to screen for contaminant-induced genetic damage. As a result, flow cytometry has been used to test for clastogenic effects of environmental toxicants in several taxa including birds (George et al. 1991; Custer et al. 1994), mammals (McBee and Bickham 1988; Bickham 1990; Biradar and Rayburn 1995; Bickham et al. 1998; Taets et al. 1998), reptiles (Bickham et al. 1988; Lamb et al. 1991), amphibians (Lowcock et al. 1997) and fish (Kocan et al. 1985; Kocan and Powell 1985; Jenner et al. 1990; Fisher et al. 1994; Easton 1997; Lingenfelser et al. 1997; Dallas et al. 1998). However, we did not detect clastogenic damage using flow cytometry on pink salmon embryos incubated in oiled gravel despite field observations and a controlled hatchery experiment that support the genetic damage hypothesis (Bue et al. 1998).

We can think of three hypotheses that might explain our inability to detect clastogenic effects in our study even though these effects may have occurred in the field, but none of these hypotheses appear to be likely. First, the complex mix of compounds that make up crude oil may produce antagonistic effects similar to those reported by Taets et al. (1998) when they examined multiple herbicides. When Taets et al. (1998) tested individual animals exposed to herbicides they found significant differences between the CV of the G1 peaks of control and exposed animals, but when the herbicides were combined, their ability to detect differences disappeared. Although the differences in the CV of the G1 peaks decreased as herbicide combinations increased, we would have been able to detect the differences they report had they existed in our study because our study had higher statistical power.

Secondly, the incubation methods used in this study may not have exposed embryos to oil in the same way as embryos were exposed to oil PWS. For example, our flushing rates and oiling concentrations may have been different than those found in PWS after the oil spill and the embryos and, in our experiments, fry were separated from the oiled gravel by a screen. However, we did observe significantly higher mortality and abnormality rates in the oiled incubators relative to the control incubators indicating that the oil was having at least large negative physiological and developmental effects. The gravel was completely coated with oil in our experiment and we used the highest dose possible according to Marty et al. (1997a). Heintz et al (1999) found survival differences for embryos incubated over gravel with crude oil levels an order of magnitude lower than the levels used in this experiment. Finally, hydrocarbon levels in embryos and fry incubated above screens, in a separate experiment using similar incubators, were not different from the levels found in embryos incubated within the gravel of the same incubators (Heintz et al 1999).

Finally, if the crude oil did affect clastogenesis in the embryos, then DNA repair might be invoked to explain our inability to detect it. Through an examination of anaphase aberrations in rainbow trout (*Salmo mykiss*), Liguori and Landolt (1985) demonstrated that DNA repair ameliorated clastogenic effects 5 d after affected animals were removed from the genotoxic challenge. However, in this study, organisms were assayed immediately upon removal from the exposure to crude oil. Further, Kocan and Powell (1985) observed elevated CV of DNA content 14 d post exposure when using flow cytometry to assay cell lines exposed to genotoxins.

Recently, other studies have also been unable to demonstrate a correlation between pollution exposure and G1 CV. Thies et al. (1996) were unable to demonstrate such a correlation in bats containing varying levels of organochlorine pesticide. In another study, no increase in G1 CV was detected in hamsters exposed to two of three herbicides tested, although the herbicide levels tested were below U.S. Environmental Protection Agency standards for drinking water (Biradar and Rayburn 1995).

Finally, Bickham et al. (1998) suggested that even without heritable mutations, pink salmon in PWS may have suffered adverse genetic effects from the *Exxon Valdez* oil spill through acute or chronic effects of oil exposure. They suggested that the oil spill, which resulted in pink salmon mortality (Bue et al. 1998), potentially produced bottlenecks that could have reduced allelic variability which in turn could threaten long term survival (Bickham and Smolen 1994) of pink salmon. For this hypothesis to be credible, the oil spill would have had to be responsible for major reductions within pink salmon populations in order for genetic drift to occur quickly. However, numbers of fish returning to oiled streams following the oil spill are within the range of those before the spill (Maki et al. 1995).

Acknowledgements

This project was funded by the *Exxon Valdez* Oil Spill Trustee Council Projects 94191 and 95191 and the Alaska Department of Fish and Game Division of Commercial Fisheries Management and Development Project 11005253. We thank Ron Heintz for providing incubator design and oiling methodology. We also thank the Prince William Sound Aquaculture Corporation for providing gametes and other support during phases of this study, and John McMahon for running flow cytometric analyses.

Literature Cited

AL-SABTI, K. (1985) Frequency of chromosomal aberrations in the rainbow trout, *Salmo gairdneri*, exposed to five pollutants. Journal of Fish Biology, 26(1), 13-19.

BICKHAM, J. W. (1990) Flow cytometry as a technique to monitor the effects of environmental genotoxins on wildlife populations. In In Situ Evaluations of Biological Hazards of Environmental Pollutants, S.S. Sandhu, W.R. Lower, F.J. de Serres, W.A. Suk, R.R. Tice, eds., (Plenum Press, New York), pp 97-108.

BICKHAM, J. W., HANKS, B. G., SMOLEN, M. J., LAMB, T. AND GIBBONS, J. W. (1988) Flow cytometric analysis of the effects of low-level radiation exposure on natural populations of slider turtles (*Pseudemys scripta*). Archives Environmental Contamination & Toxicology, 20, 837-841.

BICKHAM, J. W. AND SMOLEN, M. J. (1994) Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environmental Health Perspectives, 102(Suppl. 12), 25-28.

BICKHAM, J. W., MAZET, J. A. BLAKE, J., SMOLEN, M. J., YIGONG, L. AND BALLACHEY, B. E. (1998) Flow cytometric determination of genotoxic effects of exposure to petroleum in mink and sea otters. Ecotoxicology, 7, 191-199.

BIRADAR, D. P. AND RAYBURN, A. L. (1995) Chromosomal damage induced by herbicide contamination at concentrations observed in public water supplies. Journal of Environmental Quality, 24(6), 1222-1225.

BUE, B. G., SHARR, S., MOFFITT, S. D. AND CRAIG, A. (1996) Effects of the *Exxon Valdez oil spill* on pink salmon embryos and preemergent fry. American Fisheries Society Symposium, 18, 619-627.

BUE. B. G., SHARR, S. AND SEEB, J. E. (1998) Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the Exxon Valdez oil spill. Transactions of the American Fisheries Society, 127, 35-43.

CUSTER, T. W., BICKHAM, J. W., LYNE, T. B., LEWIS, T., RUEDAS, L. A., CUSTER, C. M. AND MELANCON, M J. (1994) Flow cytometry for monitoring contaminant exposure in black-crowned night-herons. Archives of Environmental Contamination & Toxicology, 27(2), 176-179.

DALLAS, C. E., LINGENFELSER, S. F., LINGENFELSER, J. T., HOLLOMAN, K., JAGOE, C. H., KIND, J. A., CHESSER, R. K AND SMITH, M. H. (1998) Flow cytometric analysis of erythrocyte and leukocyte DNA in fish from Chernobyl-contaminated ponds in the Ukraine. Ecotoxicology, 7, 211-219.

DANIELS, C. B. AND MEANS, J. C. (1989) Assessment of the genotoxicity of produced water discharges associated with oil and gas production using a fish embryo and larval test. Marine Environmental Research, 28, 303-307.

DARZYNKIEWICZ, Z. AND CRISSMAN, H. A. (1990) Methods in cell biology. Vol. 33. Flow cytometry. (Academic Press, New York).

EASTON, M. D. L., KRUZYNSKI, G. M., SOLAR, I. I. AND DYE, H. M. (1997) Genetic toxicity of pulp mill effluent on juvenile chinook salmon (*Onchorhynchus tshawytscha*) using flow cytometry. Water Science and Technology, 35, 347-355.

FISHER, S. K., DALLAS, C. E., JAGOE, C., SMITH, M. H., BRISBIN, I. L. AND CHESSER, R. K. (1994) Sources of error associated with sample collection and preparation of nucleated blood cells for flow cytometric analyses. Cell Biology & Toxicology, 10(3), 145-153.

FONG, A. T., DASHWOOD, R. H., CHENG, R., MATHEWS, C., FORD, B., HENDRICKS, J. D. AND BAILEY, G. S. (1993) Carcinogenicity, metabolism and KI-RAS proto-oncogene activity by 7,12-dimethylbenz[a]anthracene in rainbow trout. Carcinogenesis, 14, 629-635.

GEORGE, L. S., DALLAS, C. E., BRISBIN, I. L. JR. AND EVANS, D. L. (1991) Flow cytometric DNA analysis of ducks accumulating ^{137}Cs on a reactor reservoir. Ecotoxicology & Environmental Safety, 21, 337-347.

HENDRICKS, J. D., CHENG, R. S., SHELTON, D. W., PEREIRA, C. B. AND BAILEY, G. S.(1994) Dose-dependent carcinogenicity and frequent KI-RAS proto-oncogene activation by dietary n-nitrosodiethylmine in rainbow trout. Fundamental & Applied Toxicology, 23, 53-62.

JENNER, N. K., OSTRANDER, G. K., KAVANAGH, T. J., LIVESEY, J. C., SHEN, M. W., KIM, S. C. AND HOLMS, E. H. (1990) A flow cytometric comparison of DNA content and glutathione levels in hepatocytes of English sole (*Parophyrs vetulus*) from areas of differing water quality. Archives of Environmental Contamination & Toxicology, 19, 807-815.

KOCAN, R. M. AND POWELL, D. B. (1985) Anaphase aberrations: an in vitro test for assessing the genotoxicity of individual chemicals and complex mixtures. In Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, Vol. IV, M.D. Walters , S.S. Sandhu, J. Lewta, L. Claxton, G. Strauss, S. Nesnow, eds., (Plenum Press, New York) pp75-85.

KOCAN, R. M., POWELL, D. P. AND LIGUORI, V. M. (1985) Relationship between visible and heritable chromosome damage in trout cells and embryos. Marine Environmental Research, 17, 320-323.

LAMB, T., BICKHAM, J. W., GIBBONS, J. W., SMOLEN, M. J. AND MCDOWELL, S. (1991) Genetic damage in a population of Slider Turtles (*Trachemys scripta*) inhabiting a radioactive reservoir. Archives of Environmental Contamination and Toxicology, 20, 138-142.

LAMB, T., BICKHAM, J. W., LYNE, T. B. AND GIBBONS, J. W. (1995) The slider turtle as an environmental sentinel: multiple tissue assays using flow cytometric analysis. Ecotoxicology 4, 5-13.

LANDOLT, M. AND KOCAN, R. M. (1983) Fish cell cytogenetics: a measure of the genotoxic effects of environmental pollutants. In Aquatic Toxicology, J.O. Ngriagu, ed. (John Wiley and Sons, New York), pp 335-351.

LEE G. T., THORNTHWAITE, J. T. AND RASCH, E. M. (1984) Picogram per cell determination of DNA by flow cytometry. Analytical Biochemistry, 137, 221-226.

LIGUORI, V. M. AND LANDOLT, M. L. (1985) Anaphase aberrations: an *in vivo* measure of genotoxicity. In Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, Vol. IV, Waters M.D., Shahbeg S.S., Lewtas J., Claxton L., Strauss G., and Nesnow S., eds. (Plenum Press, New York), pp87-98.

LINGENFELSER, S. F., DALLAS, C.E., JAGOE, C. H., SMITH, M. H.,BRISBIN, I. L. JR. AND CHESSER, R. K. (1997) Variation in DNA content of blood cells of largemouth bass from contaminated and uncontaminated waters. Environmental Toxicology and Chemistry, 16(10), 2136-2143.

LONGWELL, A.C. (1977) A genetic look at fish eggs and oil. Oceanus, 20(4), 46-58.

LOWCOCK, L. A., SHARBEL, T. F. BONIN, J., OUELLET, M. RODRIGUE, M J. AND DESGRANGES, J.-L. (1997) Flow cytometric assay for in vivo genotoxic effects of pesticides in Green frogs (*Rana clamitans*). Aquatic Toxicology 38, 241-255.

MAKI, A. W., BRANNON, E. J., GILBERTSON, L. G., MOUTLON, L. L. AND SKALSKI J. R. (1995) An assessment of oil-spill effects on pink salmon populations following the *Exxon Valdez* oil spill – Part II: Adults and Escapment. In *Exxon Valdez* Oil Spill: Fate and Effects on Alaskan Waters, ASTM STP 1219, P. G. Wells, J. N. Butler and J. S. Hughes, eds. (American Society for the Testing and Materials, Philadelphia), pp 585-625.

MARTY, G. D., SHORT J. W., DOMBACH, D. M., WILLITS, N. H., HEINTZ R. A., RICE S. D., STEGEMAN J. J. AND HINTON, D. E. (1997a) Ascites, premature emergence, increased gonadal cell apoptosis and cytochrome-P451A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. Canadian Journal of Zoology, 75, 998-1007.

MARTY, G. D., HEINTZ, R. A. AND HINTON, D. E. (1997b) Histology and teratology of pink salmon larvae near the time of emergence from gravel substrate in the laboratory. Canadian Journal of Zoology, 75, 978-988.

MCBEE, K. AND BICKHAM, J. W. (1988) Petrochemical-related DNA damage in wild rodents detected by flow cytometry. Bulletin of Environmental Contamination & Toxicology, 40, 343-349.

ROY, N. K., STABILE, J., SEEB, J. E., HABICHT, C. AND WIRGIN, I. (1999) High frequency of K-*ras* mutations observed in pink salmon embryos experimentally exposed to *Exxon Valdez* crude oil. Environmental Toxicology & Chemistry 18(7):77-84.

SAS INSTITUTE INC. (1990) SAS/STAT™ User's Guide, Volume 2, Version 6. 4th edition. SAS Institute., Cary, North Carolina, USA.

TAETS, C., AREF, S. AND RAYBURN, A. L. (1998) The clastogenic potential of triazine herbicide combinations found in potable water supplies. Environmental Health Perspectives, 106,(4), 197-201.

TIERSCH, T. R. AND WACHTEL, S. S. (1993) Sources of error in screening by flow cytometry for the effects of environmental mutagens. Environmental Toxicology & Chemistry, 12, 37-42.

THIES, M. L., THIES, K. AND MCBEE, K. (1996) Organochlorine pesticide accumulation and genotoxicology in Mexican free-tailed bats from Oklahoma and New Mexico. Archives of Environmental Contamination & Toxicology, 30(2), 178-187.

VAN BENEDEN, R. J. AND OSTRANDER, G. K. (1994) Expression of oncogenes and tumor suppressor genes in teleost fishes. In Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives, D.C. Malins and G.K. Ostrander, eds. (Lewis Publishers, Boca Raton, Florida), pp 295-326.

Table 1. Percent of abnormal embryos and percent of embryos that survived to the eye stage from families incubated below the top plate in incubators with oiled and control (unoiled) gravel. We did not include data for family #2 set in these tests due to contamination in the oiled incubator of embryos seeded above the screen and no eggs were incubated in #6 incubators.

Family #	Survival (%)		Abnormality (%)	
	Oil	Control	Oil	Control
1	0.6	1.2	0.0	0.0
3	19.1	23.7	19.4	0.0
4	11.3	13.1	11.0	0.0
5	25.1	27.1	12.3	0.0
7	36.6	38.8	22.7	0.0
8	11.3	19.4	1.1	0.0
9	14.0	32.4	53.0	1.2
10	19.3	28.4	10.0	0.5
11	16.1	23.3	13.6	0.0
Mean	17.0	23.0	15.9	0.2

Table 2. Direction of expected change for each variable due to clastogens, sample means at the neural and eyed stage for control and oiled embryos for each variable, observed change in each variable, and *P*-values associated with these observations.

Variable	Expected change with treatment	Development stage	Control mean	Oiled mean	Change (O - C)	*P*-value
CV of G1	increase*	neural	4.382	4.353	-0.029	0.6725
		eyed	4.219	4.201	-0.018	0.6872
Percent of cells in S-phase	increase	neural	28.16	27.93	-0.23	0.6698
		eyed	19.33	19.15	-0.17	0.6190
G1/G2 ratio	decrease	neural	5.211	5.136	-0.75	0.7998
		eyed	10.06	10.21	+0.15	0.7215
Hyperdiploid residual width	increase	neural	3.697	3.666	-0.031	0.8920
		eyed	2.513	3.654	+0.114	0.4565

* Custer et al. (1994) found a decrease in G1 CV in night-heron chicks exposed to petroleum; all other studies with significant results found an increase with exposure.

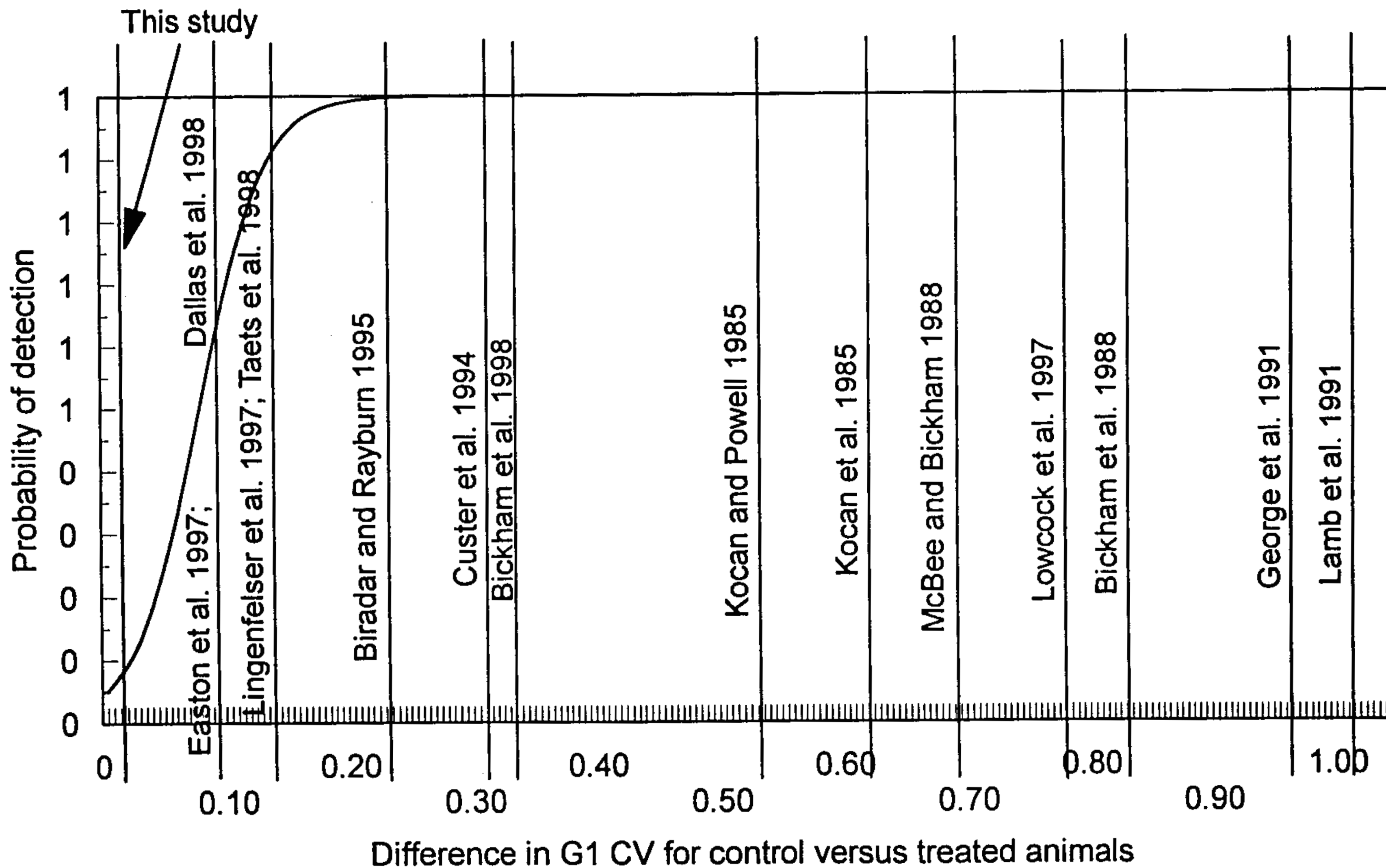
Table 3. The smallest significant differences in G1 CV (treated versus control) found in other studies using flow cytometry in relation to the power.

Study	Genotoxin	Minimum dose for which an effect of a genotoxin was detected	Animal	G1 CV of control	Smallest detected difference in G1 CV
This study	Crude oil	No effect detected with 5.6 gr/kg rock incubation substrate	Pink salmon	4.13	None detected. Had 90% chance of detecting difference of 0.14.
Kocan et al. 1985	MNNG, Benzo[a]pyrene	3.0 ug/ml, 0.20 ug/ml	Trout cell culture	3.00	0.64
Kocan and Powell 1985	Benzo[a]pyrene	0.20 ug/ml	Trout cell culture	4.49	0.55
Bickham et al. 1988	Radiation	7,950 CPM Thyac reading	Slider turtle	2.98	0.86
McBee and Bickham 1988	Petrochemical and heavy metal pollutants	Not reported	Rodents	3.23	0.71
Bickham 1990	Triethylienemelamine	0.10 mg/kg animal	Rat	Not reported	Not reported
George et al. 1991	Radiation (cesium-137)	2.52 Bq/g	Duck	2.95	0.99
Lamb et al. 1991	Radiation (cesium-137, strontium-90)	1.00 Bq/gBM, 2.24 Bq/gBM	Slider turtle	4.04	1.05
Custer et al. 1994	Petroleum, PCBs, heavy metals	Not reported	Night-heron	3.44	0.32

Table 3 (continued)

Study	Genotoxin	Minimum dose for which an effect of a genotoxin was detected	Animal	G1 CV of control	Smallest detected difference in G1 CV
Biradar and Rayburn 1995	Herbicide (atrazine)	0.23 uM	Hamster ovary cell culture	3.89 for one chromosome	0.24 for one chromosome
Easton et al. 1997	Pulp mill effuent	2 – 16%	Chinook salmon	Not reported	0.10
Lowcock et al. 1997	Pesticides (various)	Not reported	Green frog	3.20	0.81
Lingenfelser et al. 1997	Radiocesium, mercury, and PCBs	Not reported	Bass	1.61	0.15
Dallas et al. 1998	Radionuclides	Not reported	Fishes	1.19	0.10
Taets et al. 1998	Herbicide (atrazine, *simazine and cyanazine)*	0.001-0.018 ug/ml	Hamster ovary cell culture	3.06	0.15
Bickham et al. 1998	Crude oil and bunker C fuel oil	100–500 p.p.m. internal, 500 p.p.m. external	Mink and sea otters	3.52	0.35

Figure 1. Power curve demonstrating the probability of detecting differences in CV of G1 peaks between oiled and control embryos at $\alpha = 0.05$. Superimposed are the smallest differences in the CV of the G1 peak for control versus treated anaimals detected by other authors using CV of the G1 peak to detect genetic damage. For more information on these authors' experiments see Table 2.



Appendix B. Analysis of Deleterious Mutations in Pink Salmon using Haploid Androgenesis

Gary Thorgaard, Paul Wheeler and Barrie Robison
Department of Zoology
Washington State University
Pullman WA 99164-4236

Introduction: Induced haploid androgenesis was used to compare the level of deleterious mutations carried by adult male pink salmon that had been exposed to known doses of oil during their early development with the levels in control individuals. Encouraging results obtained in similar experiments using androgenetic rainbow trout and U-V treated sperm during the fall of 1994 suggested that, if differences in levels of mutations carried by these two groups existed, they might be detected by this technique.

Background Information: Androgenesis is a procedure used to produce individuals with only paternal chromosomes. In salmonid fishes, androgenesis involves treating the eggs with ionizing radiation before fertilization to destroy the maternal chromosome set. If no further treatment is applied, the irradiated eggs will produce haploid embryos with one chromosome set from the male parent and none from the female parent. Either a heat or pressure treatment can be used shortly after fertilization to block the first cleavage to produce diploid individuals with two identical sets of paternal chromosomes. Our lab has used this technique to generate clonal lines of rainbow trout for use as experimental animals. Clonal lines are made by raising androgenetic diploids to sexual maturity then making identical offspring from these individuals by androgenesis or gynogenesis depending on the sex of the original androgenetic individual (1-6).

In this study, we used haploid androgenesis as an assay to detect deleterious mutations in male pink salmon. Haploids were used rather than diploids because haploids show higher and more consistent survival, not having been heat or pressure treated. Mutations should not interfere with the egg's ability to initiate early cell cleavage. However, both dominant and recessive deleterious mutations should kill haploid embryos later in development. Only dominant mutations would kill diploid embryos with a chromosome set from each parent. Thus, an assay based on haploid androgenesis rather than normal, diploid fertilization should be considerably more sensitive. Results from a 1994 study in our lab using both androgenetic haploid and control rainbow trout indicated that this approach showed considerable promise (7). In addition, earlier studies have indicated that haploid salamanders were very sensitive to mutagenic chemicals (8) and that harmful mutations reduce survival of haploid zebrafish (9).

Androgenesis Experiments: Sperm was collected from control pink salmon males and males which had been exposed to varying doses of oil as alevins. Eggs were collected from control females. Gametes were shipped immediately from the collection site at Little Port Walter, Alaska to Washington State University, Pullman, Washington where the androgenesis experiments were conducted. Four shipments were made on the following dates: 9/12/95, 9/17/95, 9/24/95, 9/30/95, and the experiments were conducted 9/13/95, 9/19/95, 9/25/95, and 10/2/95 respectively. Transit time varied due to unpredictable weather in Alaska but every effort was made to minimize the time interval between gamete collection and androgenesis. Sperm motility was checked soon after the shipments were received to ensure that eggs were not wasted by using completely non-motile sperm. In all experiments, eggs from multiple females were

pooled and exposed to a dose of 40.0 Krads of gamma radiation using a Cobalt-60 source located at the WSU Nuclear Radiation Center, Eggs were separated into approximately equal batches for each male, fertilized and placed in a Heath-style incubator. The incubator water was recirculated and was held at a constant 9.5° C. Subsamples of eggs (mean of 84, standard deviation of 20) fertilized by each male were removed and fixed in Stockards solution 16 hours (4 cell-stage) , 16 days (streak-stage) , and 23 days (eyed stage) after fertilization. The rate of survival to each stage was later determined by examining the fixed eggs. A dissecting microscope was used to look for the presence or absence of dividing cells in the 16-hour subsamples while the 16 and 23-day subsamples were examined with the naked eye. The embryo at the two later stages is readily visible in fixed eggs. Survival was simply calculated as the number of embryos present divided by the total number of eggs in each subsample.

Statistical analysis: The data for all experiments were pooled because incubation conditions were identical. The data were analyzed with a hierarchical univariate Analysis of Variance for each developmental stage. The model for the ANOVAs consisted of treatments and individuals nested within treatments. The percent survival data were standardized to the cleavage stage and transformed with an arcsine transformation to correct for nonnormality. Means were compared using Scheffe's multiple comparisons procedure.

Results: No statistically significant differences in survival between progeny of control males and males exposed to high oil were detected. Significant differences were observed between control males and males exposed to low and medium levels of oil. However, these differences showed no trends and were likely the result of low numbers of males. Mean survival rates for experiments one, three, and four combined are given in Table 1. Experiment two was abandoned due to very low survival rates to the 4-cell stage.

		Survival Rate	
Treatment (# males)	Cleavage mean (+/-)	Streak mean (+/-)	Eyed mean (+/-)
Control (19 males)	0.456 (0.035)	0.217 (0.009)	0.074 (0.003)
Low oil (4 males)	0.200 (0.035)	0,455 (0.020)	0.007 (0.002)
Medium oil (4 males)	0.745 (0.030)	0.159 (0.018)	0.034 (0007)
High oil (16 males)	0.495 (0.031)	0.216 (0.010)	0.060 (0.003)

Table 1. Mean survival rates from experiments 1,3 and 4 combined. Standard error is given in brackets. Streak and Eyed survival are expressed as a proportion of the Cleavage survival.

The raw data from experiments one, three, and four are shown in Appendix 1. SAS outputs showing more details of the statistical analysis are provided in Appendix II.

Summary: Haploid embryo survival to all three developmental stages was strikingly similar between control males and those exposed to high levels of oil. These data indicate there are no detectable differences in survival between androgenetic haploid progeny of pink salmon exposed to oil and those not exposed. It is likely that, had more males treated with low and medium levels of oil been tested, the differences in mean survival between these treatments and the controls would have been much less.

References:

1. Parsons, J.E. and G.H. Thorgaard, 1984. Induced androgenesis in rainbow trout. J. Exp. Zool. 231: 407-412.

2. Parsons, J.E. and G.H. Thorgaard, 1985. Production of androgenetic diploid trout. J. Hered. 76: 177-181.

3. Scheerer, P.D., G.H. Thorgaard, F.W. Allendorf and K.L. Knudsen, 1986. Androgenetic rainbow trout produced from inbred and outbred sperm sources show similar survival. Aquaculture 57: 289-298.

4. Thorgaard, G.H., P.D. Scheerer, W.K. Hershberger and J.M. Myers, 1990. Androgenetic rainbow trout produced using sperm from tetraploid males show improved survival. Aquaculture 85: 215-221.

5. Scheerer, P.D., G.H. Thorgaard and F.W. Allendorf, 1991. Genetic analysis of androgenetic rainbow trout. J. Exp. Zool. 260: 382-390.

6. Young, W.P., P.A. Wheeler, R.D. Fields and G.H. Thorgaard, 1996. DNA fingerprinting confirms isogenicity of androgenetically-derived rainbow trout lines. J. Hered. 87: 77-81.

7. Thorgaard, G. H., 1994. Detection of deleterious mutations in Pink Salmon through haploid androgenesis. Project completion report to Alaska Department of Fish and Game.

8. Armstrong, J.B. and W.S. Fletcher, 1983. Assesment of mutagenic damage following ethyl methanesulonate mutagenesis in the axolotl (Ambystoma mexicanum). J. Exp. Zool, 226: 333-340.

9. Streisinger, G., C. Walker, N. Dower, D. Knauber and F. Singer, 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291: 293-296.

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
A	High	0	99	0.00	A	1	Eyed
B	High	0	54	0.00	A	1	Eyed
C	High	0	89	0.00	A	1	Eyed
D	High	0	103	0.00	A	1	Eyed
E	High	0	86	0.00	A	1	Eyed
F	High	0	91	0.00	A	1	Eyed
G	Low	0	103	0.00	A	1	Eyed
H	Low	1	101	0.01	A	1	Eyed
I	Low	0	90	0.00	A	1	Eyed
I	Low	0	77	0.00	A	1	Eyed
K	Control	0	58	0.00	A	1	Eyed
L	Control	0	62	0.00	A	1	Eyed
m	Control	0	81	0.00	A	1	Eyed
N	Control	0	83	0.00	A	1	Eyed
0	Control	3	67	0.04	A	1	Eyed
p	Control	3	95	0.03	A	1	Eyed
Q	Control	0	62	0.00	A	1	Eyed
R	Control	0	85	0.00	A	1	Eyed
S	Control	0	114	0.00	A	1	Eyed
T	Control	0	92	.0.00	A	1	Eyed
A	High	4	100	0.04	A	1	Streak
B	High	17	92	0.18	A	1	Streak
C	High	6	101	0.06	A	1	Streak
D	High	18	85	0.21	A	1	Streak
E	High	4	61	0.07	A	1	Streak
F	High	4	81	0.05	A	1	Streak
G	Low	5	75	0.07	A	1	Streak
H	Low	4	72	0.06	A	1	Streak
I	Low	14	68	0.21	A	1	Streak
J	Low	2	97	0.02	A	1	Streak
K	Control	14	68	0.21	A	1	Streak
L	Control	9	86	0.10	A	1	Streak
M	Control	8	75	0.11	A	1	Streak
N	Control	5	76	0.07	A	1	Streak
0	Control	6	69	0.09	A	1	Streak
p	Control	9	69	0.13	A	1	Streak
Q	Control	2	62	0.03	A	1	Streak
R	Control	4	80	0.05	A	1	Streak
S	Control	5	103	0.05	A	1	Streak
T	Control	11	62	0.18	A	1	Streak
A	High	36	83	0.43	A	1	Cleavage
B	High	39	61	0.64	A	1	Cleavage
C	High	40	77	0.52	A	1	Cleavage
D	High	24	90	0.27	A	1	Cleavage
E	High	18	78	0.23	A	1	Cleavage
F	High	25	82	0.30	A	1	Cleavage
G	Low	18	62	0.29	A	1	Cleavage

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
H	Low	17	76	0.22	A	1	Cleavage
I	Low	21	86	0.24	A	1	Cleavage
J	Low	7	61	0.11	A	1	Cleavage
K	Control	44	52	0.85	A	1	Cleavage
L	Control	42	72	0.58	A	1	Cleavage
M	Control	30	76	0.39	A	1	Cleavage
N	Control	13	70	0.19	A	1	Cleavage
0	Control	43	75	0.57	A	1	Cleavage
p	Control	28	63	0.44	A	1	Cleavage
Q	Control	21	79	0.27	A	1	Cleavage
R	Control	22	93	0.24	A	1	Cleavage
S	Control	17	81	0.21	A	1	Cleavage
T	Control	31	52	0.60	A	1	Cleavage
1227	High	2	63	0.03	A	3	Streak
1300	High	7	83	0.08	A	3	Streak
1259	High	8	119	0.07	A	3	Streak
1285	Control	8	113	0.07	A	3	Streak
1240	High	7	95	0.07	A	3	Streak
1235	Control	5	94	0.05	A	3	Streak
1231	Control	5	85	0.06	A	3	Streak
1270	High	8	79	0.10	A	3	Streak
1241	Control	8	70	0.11	A	3	Streak
1280	High	11	109	0.10	A	3	Streak
1282	High	5	79	0.06	A	3	Streak
1264	Control	5	59	0.08	A	3	Streak
1227	High	4	95	0.04	A	3	Eyed
1300	High	0	89	0.00	A	3	Eyed
1259	High	3	130	0.02	A	3	Eyed
1285	Control	0	85	0.00	A	3	Eyed
1240	High	2	88	0.02	A	3	Eyed
1235	Control	6	89	0.07	A	3	Eyed
1231	Control	2	102	0.02	A	3	Eyed
1270	High	3	93	0.03	A	3	Eyed
1241	Control	0	94	0.00	A	3	Eyed
1280	High	4	101	0.04	A	3	Eyed
1282	High	0	82	0.00	A	3	Eyed
1264	Control	2	71	0.03	A	3	Eyed
1227	High	39	75	0.52	A	3	Cleavage
1300	High	48	84	0.57	A	3	Cleavage
1259	High	53	108	0.49	A	3	Cleavage
1285	Control	41	111	0.37	A	3	Cleavage
1240	High	36	98	0.37	A	3	Cleavage
1235	Control	56	118	0.47	A	3	Cleavage
1231	Control	40	89	0.45	A	3	Cleavage
1270	High	53	102	0.52	A	3	Cleavage
1241	Control	57	82	0.70	A	3	Cleavage
1280	High	64	121	0.53	A	3	Cleavage

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
1282	High	29	90	0.32	A	3	Cleavage
1264	Control	32	64	0.50	A	3	Cleavage
A	High	1	66	0.02	B	1	Eyed
B	High	0	79	0.00	B	1	Eyed
C	High	0	60	0.00	B	1	Eyed
D	High	1	66	0.02	B	1	Eyed
E	High	1	70	0.01	B	1	Eyed
F	High	0	63	0.00	B	1	Eyed
G	Low	1	110	0.01	B	1	Eyed
H	Low	0	54	0.00	B	1	Eyed
I	Low	0	62	0.00	B	1	Eyed
I	Low	0	64	0.00	B	1	Eyed
K	Control	0	67	0.00	B	1	Eyed
L	Control	0	72	0.00	B	1	Eyed
M	Control	2	105	0.02	B	1	Eyed
N	Control	2	93	0.02	B	1	Eyed
0	Control	0	80	0.00	B	1	Eyed
P	Control	0	83	0.00	B	1	Eyed
Q	Control	2	68	0.03	B	1	Eyed
R	Control	0	79	0.00	B	1	Eyed
S	Control	0	78	0.00	B	1	Eyed
T	Control	0	73	0.00	B	1	Eyed
A	High	10	60	0.17	B	1	Streak
B	High	22	84	0.26	B	1	Streak
C	High	2	79	0.03	B	1	Streak
D	High	7	71	0.10	B	1	Streak
E	High	5	67	0.07	B	1	Streak
F	High	8	88	0.09	B	1	Streak
G	Low	8	65	0.12	B	1	Streak
H	Low	8	79	0.10	B	1	Streak
I	Low	6	89	0.07	B	1	Streak
J	Low	6	72	0.08	B	1	Streak
K	Control	1	69	0.01	B	1	Streak
L	Control	8	75	0.11	B	1	Streak
M	Control	10	87	0.11	B	1	Streak
N	Control	6	97	0.06	B	1	Streak
0	Control	9	70	0.13	B	1	Streak
P	Control	11	87	0.13	B	1	Streak
Q	Control	3	46	0.07	B	1	Streak
R	Control	8	78	0.10	B	1	Streak
S	Control	9	92	0.10	B	1	Streak
T	Control	10	100	0.10	B	1	Streak
A	High	14	48	0.29	B	1	Cleavage
B	High	50	83	0.60	B	1	Cleavage
C	High	15	77	0.19	B	1	Cleavage
D	High	15	63	0.24	B	1	Cleavage
E	High	12	65	0.18	B	1	Cleavage

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
F	High	24	86	0.28	B	1	Cleavage
G	Low	25	79	0.32	B	1	Cleavage
H	Low	11	45	0.24	B	1	Cleavage
I	Low	7	83	0.08	B	1	Cleavage
J	Low	9	86	0.10	B	1	Cleavage
K	Control	24	71	0.34	B.	1	Cleavage
L	Control	25	87	0.29	B	1	Cleavage
M	Control	22	69	0.32	B	1	Cleavage
N	Control	29	115	0.25	B	1	Cleavage
0	Control	15	99	0.15	B	1	Cleavage
P	Control	29	67	0.43	B	1	Cleavage
Q	Control	35	91	0.38	B	1	Cleavage
R	Control	41	93	0.44	B	1	Cleavage
S	Control	26	90	0.29	B	1	Cleavage
T	Control	61	94	0.65	B	1	Cleavage
1227	High	5	90	0.06	B	3	Streak
1300	High	12	96	0.13	B	3	Streak
1259	High	10	56	0.18	B	3	Streak
1285	Control	10	74	0.14	B	3	Streak
1240	High	11	84	0.13	B	3	Streak
1235	Control	16	95	0.17	B	3	Streak
1231	Control	14	107	0.13	B	3	Streak
1270	High		64	0.14	B	3	Streak
1241	Control	13	108	0.12	B	3	Streak
1280	High	17	122	0.14	B	3	Streak
1282	High	2	92	0.02	B	3	Streak
1264	Control	2	92	0.02	B	3	Streak
1227	High	0	71	0.00	B	3	Eyed
1300	High	0	91	0.00	B	3	Eyed
1259	High	0	72	0.00	B	3	Eyed
1285	Control	0	65	0.00	B	3	Eyed
1240	High	3	71	0.04	B	3	Eyed
1235	Control	4	92	0.04	B	3	Eyed
1231	Control	0	98	0.00	B	3	Eyed
1270	High	2	82	0.02	B	3	Eyed
1241	Control	5	107	0.05	B	3	Eyed
1280	High	3	110	0.03	B	3	Eyed
1282	High	5	98	0.05	B	3	Eyed
1264	Control	1	69	0.01	B	3	Eyed
1227	High	54	86	0.63	B	3	Cleavage
1300	High	47	83	0.57	B	3	Cleavage
1259	High	32	77	0.42	B	3	Cleavage
1285	Control	53	103	0.51	B	3	Cleavage
1240	High	34	67	0.51	B	3	Cleavage
1235	Control	36	93	0.39	B	3	Cleavage
1231	Control	49	98	0.50	B	3	Cleavage
1270	High	53	116	0.46	B	3	Cleavage

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
1241	Control	44	113	0.39	B	3	Cleavage
1280	High	61	90	0.68	B	3	Cleavage
1282	High	35	79	0.44	B	3	Cleavage
1264	Control	47	124	0.38	B	3	Cleavage
1188	Medium	3	107	0.03	A	2	Eyed
1193	Medium	1	110	0.01	A	2	Eyed
1194	High	1	60	0.02	A	2	Eyed
1202	Medium	2	63	0.03	A	2	Eyed
1196	Medium	1	80	0.01	A	2	Eyed
1197	High	1	93	0.01	A	2	Eyed
1204	Control	2	78	0.03	A	2	Eyed
1208	Control	4	90	0.04	A	2	Eyed
1199	High	0	77	0.00	A	2	Eyed
1200	Control	0	62	0.00	A	2	Eyed
1206	Control	1	54	0.02	A	2	Eyed
1188	Medium	72	98	0.73	A	2	Cleavage
1193	Medium	46	58	0.79	A	2	Cleavage
1194	High	34	76	0.45	A	2	Cleavage
1202	Medium	60	84	0.71	A	2	Cleavage
1196	Medium	57	88	0.65	A	2	Cleavage
1197	High	65	80	0.81	A	2	Cleavage
1204	Control	59	97	0.61	A	2	Cleavage
1208	Control	60	77	0.78	A	2	Cleavage
1199	High	82	109	0.75	A	2	Cleavage
1200	Control	0	64	0.00	A	2	Cleavage
1206	Control	64	87	0.74	A	2	Cleavage
1188	Medium	26	119	0.22	A	2	Streak
1193	Medium	13	64	0.20	A	2	Streak
1194	High	14	88	0.16	A	2	Streak
1202	Medium	4	87	0.05	A	2	Streak
1196	Medium	13	94	0.14	A	2	Streak
1197	High	9	82	0.11	A	2	Streak
1204	Control	11	51	0.22	A	2	Streak
1208	Control	9	66	0.14	A	2	Streak
1199	High	5	86	0.17	A	2	Streak
1200	Control	0	66	0.00	A	2	Streak
1206	Control	20	82	0.24	A	2	Streak
1188	Medium	2	65	0.03	B	2	Eyed
1193	Medium	0	68	0.00	B	2	Eyed
1194	High	1	68	0.01	B	2	Eyed
1202	Medium	2	79	0.03	B	2	Eyed
1196	Medium	0	69	0.00	B	2	Eyed
1197	High	0	90	0.00	B	2	Eyed
1204	Control	3	86	0.03	B	2	Eyed
1208	Control	4	81	0.05	B	2	Eyed
1199	High	1	98	0.01	B	2	Eyed
1200	Control	1	110	0.01	B	2	Eyed

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
1206	Control	1	60	0.02	B	2	Eyed
1188	Medium	68	76	0.89	B	2	Cleavage
1193	Medium	49	61	0.80	B	2	Cleavage
1194	High	61	95	0.64	B	2	Cleavage
1202	Medium	55	96	0.57	B	2	Cleavage
1196	Medium	59	79	0.75	B	2	Cleavage
1197	High	55	69	0.80	B	2	Cleavage
1204	Control	83	113	0.73	B	2	Cleavage
1208	Control	64	80	0.80	B	2	Cleavage
1199	High	87	118	0.74	B	2	Cleavage
1200	Control	0	68	0.00	B	2	Cleavage
1206	Control	77	97	0.79	B	2	Cleavage
1188	Medium	7	82	0.09	B	2	Streak
1193	Medium	5	57	0.09	B	2	Streak
1194	High	9	107	0.08	B	2	Streak
1202	Medium	2	74	0.03	B	2	Streak
1196	Medium	12	93	0.13	B	2	Streak
1197	High	11	71	0.15	B	2	Streak
1204	Control	5	89	0.06	B	2	Streak
1208	Control	6	40	0.15	B	2	Streak
1199	High	10	91	0.11	B	2	Streak
1200	Control	0	58	0.00	B	2	Streak
1206	Control	14	115	0.12	B	2	Streak
1188	Medium	1	22	0.05	C	2	Eyed
1193	Medium	2	24	0.08	C	2	Eyed
1194	High	1	45	0.02	C	2	Eyed
1202	Medium	1	30	0.03	C	2	Eyed
1196	Medium	0	27	0.00	C	2	Eyed
1197	High	1	39	0.03	C	2	Eyed
1204	Control	2	105	0.02	C	2	Eyed
1208	Control	5	90	0.06	C	2	Eyed
1199	High	2	65	0.03	C	2	Eyed
1200	Control	0	91	0.00	C	2	Eyed
1206	Control	3	98	0.03	C	2	Eyed
1188	Medium	72	83	0.87	C	2	Cleavage
1193	Medium	106	135	0.79	C	2	Cleavage
1194	High	45	91	0.49	C	2	Cleavage
1202	Medium	69	120	0.58	C	2	Cleavage
1196	Medium	92	114	0.81	C	2	Cleavage
1197	High	75	118	0.64	C	2	Cleavage
1204	Control	119	158	0.75	C	2	Cleavage
1208	Control	65	95	0.68	C	2	Cleavage
1199	High	66	84	0.79	C	2	Cleavage
1200	Control	2	103	0.02	C	2	Cleavage
1206	Control	101	140	0.72	C	2	Cleavage
1188	Medium	20	105	0.19	C	2	Streak
1193	Medium	10	87	0.11	C	2	Streak

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
1194	High	5	121	0.04	C	2	Streak
1202	Medium	4	115	0.03	C	2	Streak
1196	Medium	19	136	0.14	C	2	Streak
1197	High	6	92	0.07	C	2	Streak
1204	Control	12	127	0.09	C	2	Streak
1208	Control	9	92	0.10	C	2	Streak
1199	High	27	125	0.22	C	2	Streak
1200	Control	0	94	0.00	C	2	Streak
1206	Control	14	107	0.13	C	2	Streak

Appendix II

I

The SAS System 2

STAGE=Cleavage

General Linear Models Procedure

Dependent Variable: TRANSURV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	38	18.77818341	0.49416272	9.02	0.0001
Error	46	2.51979267	0.05477810		
Corrected Total	84	21.29797608			

R-Square	C.V.	Root MSE	TRANSURV Mean
0.881689	16.25641	0.2340472	1.4397229

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	2.76730419	1.38365210	25.26	0.0001
INDIV(TRT)	36	16.01087921	0.44474664	8.12	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	2.46698761	1.23349380	22.52	0.0001
INDIV(TRT)	36	16.01087921	0.44474664	8.12	0.0001

-- STAGE=Cleavage --

General Linear Models Procedure

Scheffe's test for variable: TRANSURV

...... NOTE: This test controls the type I experimentwise error rate but generally has a higher type II error rate than Tukey's for all pairwise comparisons.

Alpha= 0.05 Confidence= 0.95 df= 46 MSE= 0.054778
Critical Value of F= 3.19958

Comparisons significant at the 0.05 level are indicated by '***'.

	TRT Comparison	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit	
High	- Control	-0.01821	0.11729	0.25279	
High	- Low	0.41985	0.65186	0.88388	***
Control	- High	-0.25279	-0.11729	0.01821	
Control	- Low	0.30618	0.53457	0.76297	***
Low	- High	-0.88388	-0.65186	-0.41985	***
Low	- Control	-0.76297	-0.53457	-0.30618	***

-------- -------- --- STAGE=Streak ----------------------------------

General Linear Models Procedure

Dependent Variable: TRANSURV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	38	10.46978449	0.27552064	2.38	0.0027
Error	46	5.33511847	0.11598084		
Corrected Total	84	15.80490296			

	R-Square	C.V.	Root MSE	TRANSURV Mean
	0.662439	34.26395	0.3405596	0.9939298

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	2.87720864	1.43860432	12.40	0.0001
INDIV(TRT)	36	7.59257585	0.21090488	1.82	0.0280

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	2.66661871	1.33330936	11.50	0.0001
INDIV(TRT)	36	7.59257585	0.21090488	1.82	0.0280

The SAS System 9

------------------- ------------- STAGE=Streak --------------------------------

General Linear Models Procedure
Scheffe's test for variable: TRANSURV

...... NOTE: This test controls the type I experimentwise error rate but generally has a higher type II error rate than Tukey's for all pairwise comparisons.

Alpha= 0.05 Confidence= 0.95 df= 46 MSE= 0.115981
critical Value of F= 3.19958

Comparisons significant at the 0,05 level are indicated by '***'.

	TRT Comparison	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit	
Low	- High	O.23418	0.57178	0.90939	***
Low	- Control	0.32074	0.65308	0.98541	***
High	- Low	-0.90939	-0.57178	-O.23418	***
High	- Control	-0.11588	0.08129	0.27846	
Control	- Low	-0.98541	-0.65308	-0.32074	***
Control	- High	-0.27846	-0.08129	0.11588	

------------------ ------- STAGE=Eyed ---------------------------------

General Linear Models Procedure

Dependent Variable: TRANSURV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	38	2.92812923	0.07705603	1.46	0.1108
Error	46	2.43145320	0.05285768		
Corrected Total	84	5.35958243			

	R-Square	C.V.	Root MSE	TRANSURV	Mean
	0.546335	101.5708	0.2299080		0.2263525

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	0.14550664	0.07275332	1.38	0.2627
INDIV(TRT)	36	2.78262259	0.07729507	1.46	0.1115

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	2	0.13603815	0.06801908	1.29	0.2859
INDIV(TRT)	36	2.78262259	0.07729507	1.46	0.1115

---------------------------------- STAGE=Eyed ----------------------------------

General Linear Models Procedure
Scheffe's test for variable: TRANSURV

...... NOTE: This test controls the type I experimentwise error rate but generally has a higher type II error rate than Tukey's for all pairwise comparisons.

Alpha= 0.05 Confidence= 0.95 df= 46 MSE= 0.052858
Critical Value of F= 3.19958

Comparisons significant at the 0.05 level are indicated by '***'.

	TRT Comparison	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit
High	- Control	-0.13090	0.00221	0.13532
High	- Low	-0.08506	0.14286	0.37077
Control	- High	-0.13532	-0.00221	0.13090
Control	- Low	-0.08371	0.14065	0.36500
Low	- High	-0.37077	-0.14286	0.08506
Low	- Control	-0.36500	-0.14065	0.08371

Appendix C. An Initial Evaluation of Molecular Genetic Damage to Oiled Pink Salmon Populations in Prince William Sound.

BY:
ISAAC WIRGIN AND JOSEPH STABILE
INSTITUTE OF ENVIRONMENTAL MEDICINE
NEW YORK UNIVERSITY MEDICAL CENTER
LONG MEADOW ROAD
TUXEDO, NEW YORK 10987

INTRODUCTION

Extensive genetic damage to natural populations (McMahon et al. 1990; Wirgin et al. 1989) and domesticated strains of fish (Hendricks et al. 1994) by polycyclic aromatic hydrocarbons (PAHS) exposures has been previously documented. DNA damage in these species has been associated with hepatic carcinogenesis and as such its effects are believed to be single generational. However, recent evidence suggests that genetic damage by xenobiotics to natural populations may also be transmitted intergenerationally, and thus effects from exposure may be persistent and devastating to the long term viability of natural populations (Prince and Cooper 1995; Wirgin et al. 1992).

It has been demonstrated that pink salmon populations from *Exxon Valdez* oiled streams exhibit significantly increased embryo mortality compared to populations from adjacent non-oiled streams and that this effect was persistent over several generations (Bue et al. In press). Similarly, pink salmon embryos experimentally oiled under controlled laboratory conditions demonstrated reduced survivorship compared to cohorts reared under non-oiled conditions. Multigenerational persistence of this toxic effect suggested that oil populations have suffered heritable genetic damage. Evidence suggestive of DNA damage to these populations have included 1) elevated levels of exposure to mutagenic PAH compounds (Collier et al. in press), 2) increased levels of cytochrome P4501A (CYP1A) enzyme activity. CYP1A activity is inducible by PAH exposure and is required to convert environmental PAHs to reactive metabolites which adduct to and damage DNA. Induced CYP1A activity was found in pink salmon (Wiedmer et al. in press) and benthic fish species (Collier et al. in press) from oiled sites. We hypothesized that reduced survivorship of pink salmon embryos in oiled populations results from molecular genetic damage at loci critical to normal development. However, little is known regarding the molecular aspects of development in salmon species, and genes critical to development of young life stages have yet to be identified. Thus, direct investigations of genetic variation or levels of expression of genes implicated in normal early development could not be evaluated at this time. Future studies will identify these genes and compare their structure and levels of expression between oiled and non-oiled populations.

Instead, we focused on determining if overall levels of molecular genetic damage are elevated by experimental exposure to aged Exxon Valdez oil in pink salmon embryos. Genetic loci to be investigated include the cytochrome b gene in mitochondrial DNA (mtDNA) and the K-*ras*

oncogene in the nuclear genome. DNA repair in mtDNA is not as efficient as in nuclear DNA and therefore may exhibit higher levels of nucleotide change than seen in single copy nuclear DNA genes. Cytochrome b sequence is highly conserved among vertebrate taxa and does not generally exhibit high levels of intraspecific evolutionary change. However, recent studies of cytochrome b sequence in voles exposed to radiation at Chernobyl revealed approximately 30 fold higher levels of variability in exposed compared to nearby unexposed populations for several generations after the exposure experience (Schmidt 1996). In fact, levels of nucleotide sequence change among some individual voles from exposed populations was higher than between some rodent species.

In contrast, the K-*ras* oncogene has been demonstrated to be highly sensitive to PAH induced mutation at selected codons within the first and second exons of the gene in a multitude of vertebrate taxa including several fish species (Wirgin et al. 1989; McMahon et al. 1990, Hendricks et al. 1994). Studies in environmentally exposed Atlantic tomcod and winter flounder from PAH polluted sites along the Atlantic coast of North America have demonstrated the sensitivity of this gene locus to environmental insult. Furthermore, studies in chemically treated domesticated rainbow trout have indicated that DNA damage at this locus is dose dependent in this model.

METHODS

DNA extraction and PCR amplifications

A total of 24 embryos and 20 parents were examined for nucleotide sequence variation within the mtDNA cytochrome b gene. DNA from 12 control embryos and 12 experimentally oiled embryos was provided by J. Seeb. Total DNA was extracted from parental liver tissue with C-Tab buffer (Saghai-Maroof et al. 1984) as described by Wirgin et al. (1990). DNA was purified following incubations by standard phenolchloroform extractions and alcohol precipitations.

The cytochrome B sequence was PCR amplified using the forward primer, 5' - gftagatcctgtctcatgaag - 3' (nucleotide #s 136-156) and reverse complimentary primer 5' - ctcccgtgaggacaaatgtc - 3' (nucleotide #s 356-337). The PCR reaction mix contained 2.5 µl of 10 x buffer (Promega), 1.5 µl of 25 mM MgCl,, 0.125 µl of each dNTP, 1.0 µl of each primer (30µM), 10-100 ng of template DNA and double distilled water to a final reaction volume of 25 µl. 1.5-2.5 U of Taq I polymerase (Promega) was added after the reaction mix was denatured for 5 min at 94°C. Amplification was performed in 35 cycles as follows; denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min; these cycles were followed by a final 7 min extension at 72°C.

Sequence analysis of the mtDNA cytochrome B gene

The PCR products were purified in 2.0% low melting point agarose gels (Nu-Sieve GTG, FMC Corp.), the 230 bp cytochrome b fragment was excised in an agarose plug and sequenced directly (Kretz and O'Brien, 1993) with ^{35}S using the CyclistTM Taq DNA Sequencing Kit (Stratagene), at an annealing temperature of 62°C. All samples were sequenced using the forward primer described above and a subset was sequenced with the reverse primer. DNA sequences were resolved in 6% polyacrylamide/7M urea denaturing gels. The gels were air-dried on glass plates and exposed to X-ray film for 1-5 days.

RESULTS AND DISCUSSION

Our DNA sequence for cytochrome b in pink salmon is identical to that of Seeb et al. (unpublished data) with the exception of a single nucleotide insertion (G) at nucleotide #310. Screening of a 141 base pair fragment (nucleotides 175- 316) among experimentally oiled embryos, non-oiled embryos, and their parents revealed one polymorphic nucleotide site (#268) (Figure 1). All twelve of the control embryo specimens (non-oiled) from incubator #2 and 15 parents of these crosses were monomorphic for a C nucleotide at site #268. In contrast, 5 of the 12 oiled embryos, had a T nucleotide at site #268, while the other seven oiled embryos had the normal C allele (Table 1). We have yet to characterize mtDNA genotypes in the remaining 5 parents. Additionally, preliminary results suggest that several of the control and oiled embryos exhibit heteroplasmy (individual tissues that show more than one mtDNA genotype) at this polymorphic nucleotide site. Confirmation of this observation awaits additional sequencing experiments.

These preliminary results indicate that there are significant differences ($p < 0.01$) in the frequencies of mtDNA genotypes between oil-treated and non-treated embryos. Since, the variant genotype was only observed in experimentally oiled embryos it suggests that exposure to *Exxon Valdez* oil initiated the DNA alteration. Characterization of mtDNA genotypes in the remaining five parents will confirm this finding.

Mitochondrial DNA is maternally inherited and therefore all progeny of a single female would be expected to share identical mtDNA genotypes. However, we observed heterogeneity of genotypes among progeny suggesting that some additional exogenous factor was operative. Furthermore, the cytochrome b gene is highly conserved at the intraspecific level. This would also suggest that all progeny of a single female should share identical mtDNA genotypes. Therefore, the most plausible explanation of the high frequency of variant genotypes in the experimental group is the oil treatment.

These results are unexpected in that variation was only observed at a single nucleotide site, yet the frequency of variants was high (42%) among the experimentally oiled cohorts. Additionally, all variants exhibited a C to T transition. There is no other evidence in the literature for single nucleotide "hotspots" for mutational change in the mtDNA genome in response to toxicant exposure. However, xenobiotically sensitive genes have been identified in the nuclear genome which exhibit a high frequency of nucleotide change in response to PAH exposure. For example, the spectra of mutational change at the K-*ras* locus is often species, tissue, and chemical specific (Hendricks et al. 1994). In additional studies, we will examine the frequency and spectra of mutational change at K-*ras* as an additional marker to confirm that genetic change in oiled pink salmon is frequent and is not restricted to the mtDNA genome.

LITERATURE CITED

Bue, B.G., S. Sharr, G.D. Miller, and J.E. Seeb. In press. Evidence of genetic damage in pink salmon inhabiting Prince William Sound, Alaska three generations after the *Exxon Valdez* oil spill.

Collier, T.K., M.M. Krahn, C.A. Krone, L.L. Johnson, M.S. Myers, S-L Chan, and U. Varanasi. In press. Oil exposure and effects in subtidal fish following the *Exxon Valdez* oil spill.

Hendricks. J.D., R. Cheng, D.W. Shelton, C.B. Pereira, and G.S. Bailey. 1994. Dosedependent carcinogenecity and frequent Ki-*ras* proto-oncogene activation by dietary N-nitrosodiethylamine in rainbow trout. Fund. Appl. Toxicol. 23:53-62.

Kretz, K. A., and J. S. O'Brien. 1993. Direct sequencing of polymerase chain reaction products from low melting temperature agarose. Methods Enzymol. 218: 72-79.

McMahon, G.L., L.J. Huber, M.J. Moore, J.J. Stegeman, and G.N. Wogan. 1990. Mutations in c-Ki-*ras* oncogenes in diseased livers of winter flounder from Boston Harbor. Proc. Natl. Acad. Sci. USA 87:841-845.

Prince, R., and K.R. Cooper 1995. Comparisons of the effects of 2,3,7,8tetrachlorodibenzo-p-dioxin on chemically impacted and nonimpacted subpopulations of Fundulus heteroclitus. 1. TCDD toxicity in embryos and adults of chemically impacted and nonimpacted subpopulations of Fundulus heteroclitus. Environ. Toxicol Chem. 14:579-588.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen and R.W. Allard, 1984 Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Scl. USA 81: 8014-8018.

Schmidt, K. Unnatural evolution. 1996. New Scientist 2019:34-37.

Wiedmer, M., M.J. Fink, J.J. Stegeman, R. Smolowitz, G.D. Marty, and D.E. Hinton. In press. Cytochrome P450 induction and histopathology in pre-emergent pink salmon from oiled streams in Prince William Sound, Alaska.

Wirgin, I., D. Currie, and S.J. Garte. 1989. Activation of the K-*ras* oncogene in liver tumors of Hudson River tomcod. Carcinogenesis 10:2311-2315.

Wirgin, I.I., G.-L. Kreamer, C. Grunwald, K. Squibb, S.J. Garte, and S. Courtenay. 1992. Effects of prior exposure history on cytochrome P4501A MRNA induction by PCB congener 77 in Atlantic tomcod. Mar. Environ. Res. 34:103-108.

Figure 1. Comparison of mtDNA cytochrome b sequences from #1 - control embryo, #1 - oiled embryo and OGBO8. Alignment begins at position 175 of OGBO8. Position of the polymorphic nucleotide site is highlighted in boldface.

	175
1-Control	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc
1-Oiled	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc
OGB08	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc

	225
1-Control	gtgttagggt ggcgttatca acggagaatc cgcctcaaat cca**t**tgtact
1-Oiled	gtgttagggt ggcgttatca acggagaatc cgcctcaaat cca**c**tgtact
OGB08	gtgttagggt ggcgftatca acggagaatc cgcctcaaat cca**t**tgtact

	275
1-Control	agggcgccgc ccacataggg aacagcggaa agaaggtttgt
1-Oiled	agggcgccgc ccacataggg aacagcggaa agaaggtttgt
OGB08	agggcgccgc ccacataggg aacagcggaa agaag-tttgt

Table 1 MtDNA cytochrome b genotype characterization for experimentally treated pink salmon embryos and their parents:

A. Control embryos from incubator 2.
B. Oiled embryos from incubator 2.
C. Parents of embryos in the second oiling experiment.

A.

Control Incubator 2	Mutant C	Normal T
1		+
2		+
3		+
4		+
5		+
6		+
7		+
8		+
9		+
10		+
11		+
12		+

B.

Oiled Incubator 2	Mutant C	Normal T
1	+	
2	+	
3		+
4		+
5		+
6	+	
7		+
8		+
9	+	
10		+
11		+
12	+	

C.

Parents

2nd Oiling Experiment	Mutant C	Normal T
Males		
61		+
62		+
63		+
64		
65		+
66		+
67		
68		+
69		+
70		+
Females		
135		+
136		
137		+
138		+
139		+
140		+
141		+
142		
143		
144		+

Appendix D. Use of Microsatellites in Two Genetic Studies of Alaskan Salmon (*Oncorhynchus spp.*)

Brief Summary of Results

Jeff Olsen

University of Washington School of Fisheries
Marine Molecular Biotechnology Laboratory
Seattle, Washington, and

Alaska Department of Fish and Game
Genetics Laboratory
Anchorage, Alaska

(This subproject was designed to detect microsatellite variation in salmonids, in part, for use in mutation screens for Project 95191; and the work was conducted by Graduate Intern Jeff Olsen as a Ph.D. student project. Study 1 below was primarily conducted at the University of Washington and was funded in part by Alaska Department of Fish and Game. Study 2 actually preceded Study 1 and was Alaska Department of Fish and Game where Mr. Olsen identified four microsatellite loci that resolved well in pink salmon, and this study was funded in total by Project 95191.)

Introduction

In June of 1995 the Alaska Department of Fish and Game (ADFG) Genetics Lab initiated two studies using microsatellites. The goal of study one was to assess interspecific priming of various microsatellite primer pairs in Pacific salmon. This was done by completing a broad multispecies screening of 35 salmonid microsatellite primer pairs. These primers, developed from six different salmonid taxa, were tested in five species of *Oncorhynchus* and *Salmo salar* (see Olsen et al. submitted and references therein).

The second study was part of a larger research project aimed at describing the mutagenic effects of oiling on Prince William Sound (PWS) pink salmon (*Oncorhnychus gorbuscha*) exposed to the Exxon Valdez Oil Spill (EVOS). This pilot study was done using four microsatellites described in study one to test for significant loss of heterozygosity (LOH) (see Hahn et al. 1995) in oil exposed pink salmon.

Study 1

Overview

Microsatellite screening was initiated at the ADFG Genetics laboratory in Anchorage Alaska where nine loci (FGT1, Oneμ1, Oneμ11, Oneμ14, Ots2, Ots6, Ssa4, μSat60, μSat73) were tested in four species of Pacific salmon (chinook, chum, pink, sockeye). The screening was completed at the University of Washington Marine Molecular Biotechnology (UW-MMBL). Results of this study and a summary of methods were included in a manuscript submitted to *Marine Molecular Biology and Biotechnology* (see Olsen et al. submitted).

Results and Discussion

Table 1 summarizes the PCR annealing temperature, quality of the PCR product (as described in Figure 1) and the estimated allelic range for each species ~~and~~locus combination. The quality of some amplifications, particularly those receiving a grade of 3, may be improved by increasing the annealing temperature. The source species for each primer pair was included as a positive control with the exception of the three brown trout-derived loci. The positive control received a score of 3-5 in eight instances (positive control data for Sfo8, 12, 18, and 23 not shown). This occurred three times in steelhead (see Table 1 in Wenburg et al. submitted) and brook trout (*Salvelinus fontinalis*), and twice in Atlantic salmon. In each case product quality was no better in the other species. As expected, the highest percentage of quality grades 1 and 2 occurred in those species from which some of the microsatellites were developed.

Table 2 summarizes PCR product quality for each species. The loci receiving a quality grade of 1 or 2 are summarized for each species as a percent of total loci scored. This value ranged from 31% (chum salmon) to 63% (chinook salmon). Only pink and chum salmon had fewer than 50% of the loci scoring 1 or 2. Between 57% and 74% of the loci given a quality grade of 1 or 2 also had an allelic range of 1 or 2 (60-180 b). In chinook, coho, sockeye, chum and Atlantic salmon, this value was greater than 65%.

Our screening of microsatellites in Pacific salmon demonstrates that sequence conservation in priming regions often permit inter-specific exchange of primers. These results are supported by previous findings (e.g. McConnell et al 1995; Morris et al. 1996), suggesting microsatellite based genetic studies of Pacific salmon are possible using existing primers. The high cost and time associated with creating microsatellite libraries and designing primers need not be a constraint. Further, our data should be helpful in directing researchers to useful species/primer combinations, reducing development costs associated with primer testing.

By exchanging primers across species of salmon we are assuming they amplify homologous loci. Evidence supporting this assumption has been found in salmonid fishes, mammals and sea turtles (Morris et al. 1996, Forbes et al. 1995, Fitzsimmons et al. 1995, Pepin et al. 1995). However, final verification will require sequencing of the PCR product. This is of particular importance when conducting phylogenetic surveys across taxa (Forbes et al. 1995, Estoup et al. 1995).

In some instances the degree of complementarity between a primer and the microsatellite flanking sequence may vary among alleles within a species. In extreme cases some alleles will not amplify. The presence of "null" alleles can be inferred through population level screening and testing for departures from Hardy-Weinberg equilibrium (Callen et al. 1993). Using this approach we did not see evidence of null alleles in the six loci screened in coho chinook and sockeye. Likewise, Wenburg et al. (this issue) did not see evidence of null alleles in the loci screened in steelhead and cutthroat. However, in an earlier study, we did observe evidence of one or more null alleles at microsatellite Ssa293 in sockeye (Bentzen and Olsen, unpublished data).

Study 2

Overview

This study consisted of two phases. In phase one we compared heterozygosity at four microsatellite loci among oiled and control pink salmon using DNA extracted from heart tissue (methods of DNA extraction, microsatellite amplification via Polymerase Chain Reaction (PCR), and fragment analysis using the Applied Biosystems (ABI) 373A in GeneScan mode are

described in detail in Olsen et al submitted). The four loci used included Oneμ1, Oneμ11, μSat60 μSat73.

In phase two we compared heterozygosity at three loci (Oneμ1, Oneμ11, μSat60) among four tissues (heart, liver, kidney and spleen) from oiled pink salmon. This was done to assess variation in heterozygosity at each locus/tissue combination that may have resulted from oil exposure during tissue differentiation at embyrogenesis.

Results and Discussion

Results of phase one are summarized on table 3. Oiled and control individuals were monomorphic for Oneμ1, Oneμ11 and Sat73. The microsatellite μSat60 was polymorphic, however the level heterozygosity among oiled and control individuals did not appear significantly different. Examples of electropherograms showing μSat60 alleles from five individuals are shown in figure 1. In phase two, only μSat60 was polymorphic in the four tissue types. Oneμ1 and Oneμ11 were monomorphic in all individuals regardless of tissue type. The level of heterozygosity at μSat60 did not appear significantly different among tissues (Table 4). Examples of electropherograms showing the Oneμ11 allele from three tissues samples from one individuals are shown in figure 2.

Results from this pilot study suggest a much larger battery of more highly polymorphic microsatellites would be needed to enhance likelihood of detecting LOH.

Acknowledgements

We thank the EVOS trustee council for providing financial support for studies one and two. Work carried out by JBO while at the University of Washington was funded in part by a Sea Grant award to Dr. Paul Bentzen. Tissue for DNA analyses were provided by ADF&G genetics personnel, Dr. Paul Moran of the National Marine Fisheries Service, Chuck Pevan of the Chelan County PUD, Carol Woody of the National Biological Service, and Adrian Spidle of the University of Washington.

References

Callen, D.F., Thompson, A.D., Shen, Y., Phillips, H.A., Richards, R.I., Mulley, J.C., and Sutherland, G.R. (1993). Incidence and origin of "null" alleles in the (AC)n microsatellite markers. *Am J Hum Genet* 52:922-927.

Estoup, A., Tailliez, C., Cornuet, J.M., and Solignac, M. (1995). Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifer* and *Bombus terrestris* (Apidae). *Mol Biol Evol* 12(6):1074-1084.

FitzSimmons, N.N., Moritz, C., and Moore, S.S. (1995). Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Mol Biol Evol* 12(3):432-440.

Forbes, S.H., Hogg, J.T., Buchanan, F.C., Crawford, A.M., and Allendorf, F.W. (1995). Microsatellite evolution in congeneric mammals: domestic and bighorn sheep. *Mol Biol Evol* 12(6):1106-1113.

Hahn, M., Matzen, S.E., Serth, J., Pingoud, A. (1995). Semiautomated quantitative detection of loss of heterozygosity in tumor suppressor gene *p53*. *Biotechniques* 18(6):1040-1047.

McConnell, S.K., O'Reilly, P., Hamilton, L., Wright, J.M., and Bentzen, P. (1995a). Polymorphic microsatellite loci from Atlantic salmon (*Salmo salar*): genetic differentiation of north American and European populations. *Can J Fish Aquat Sci* 52:1863-1872.

Morris, D.B., Richard, K.R., and Wright, J.M. (1996). Microsatellites from rainbow trout (*Oncorhynchus mykiss*) and their use for genetic study of salmonids. *Can J Fish Aquat Sci* 53:120-126.

Olsen, J.B., Wenburg, J.K., and Bentzen, P. (1996). Semi-automated multilocus genotyping of Pacific salmon (*Oncorhynchus* spp.) using microsatellites. *Mol Mar Biol Biotechnol* (submitted).

Pepin, L., Amigues, Y., Lepingle, A., Berthier, J., Bensiad, A., and Vaiman, D. (1995). Sequence conservation of microsatellites between *Bos taurus* (cattle), and *Capra hircus* (goat) and related species. Examples of use in parentage testing and phylogeny analysis. *Heredity* 74:53-61.

Wenburg, J., Olsen J., and Bentzen P. (XXXX). Multiplexed systems of microsatellites for genetic analysis in coastal cutthroat trout (*Oncorhynchus clarki clarki*) and steelhead (*Oncorhynchus mykiss*). *Mol Mar Biol Biotechnol* (submitted)

Table 1. Salmonid microsatellite screening results. Amplification results are coded as follows: (T) is the PCR annealing temperature; (Q) indicates the quality of the PCR amplification[a]; (S) indicates the approximate size range (bases) of the PCR product[b]. The loci used in multiplex development are shown in bold for chinook, coho, and sockeye

Microsat. Locus	Chinook (T)-(Q)-(S)	Coho (T)-(Q)-(S)	Sockeye (T)-(Q)-(S)	Pink (T)-(Q)-(S)	Chum (T)-(Q)-(S)	Atlantic (T)-(Q)-(S)
Fgt1	(56)-2-3,4	(56)-4-3,4	(56)-2-3	(56)-2-2	(56)-3-4	(56)-4-3,4
Omy77	(50)-2-2	(50)-2-2	(50)-2-1	(50)-5	(50)-5	(50)-2-1
Omy78	(55)-4-1	(55)-4-1	(55)-4-1	(55)-4-1	(55)-4-1	(55)-4-1
Omy87	(55)-4-1,2	(55)-4-2,3	(55)-3-2	(55)-3-1,2	(55)-4-3,4	(55)-4-1,2
Omy207	(56)-1-1	(56)-1-1	(56)-5	(53)-5	(53)-5	(53)-1-1
Omy293	(55)-5	(55)-5	(55)-5	(55)-5	(55)-5	(55)-5
Omy325	(58)-2-1	(58)-2-1,2	(60)-2-2	(55)-2-3	(55)-3-2,3	(58)-2-2,3
Oneμ1	(58)-5	(58)-2-2	(58)-2-1	(58)-2-1	(58)-5	(58)-2-1,2
Oneμ2	(58)-5	(58)-2-2,3	(58)-2-3,4	(57)-5	(57)-2-4	(58)-2-3,4
Oneμ8	(58)-2-2	(55)-2-3	(58)-2 -3	(55)-2-4	(55)-2-3	(55)-5
Oneμ10	(57)-5	(57)-5	(57)-2-1,2	(57)-5	(57)-5	(57)-5
Oneμ11	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2
Oneμ14	(58)-2-3	(58)-2-3,4	(58)-2-2	(58)-5	(58)-5	(58)-2-3
Ots1	(50)-1-3	(50)-1-3	(50)-5	(50)-2-3	(50)-2-2	(50)-1-3
Ots2	(48)-2-1	(48)- 2-1	(48)-5	(50)-5	(50)-5	(48)-5
Ots3	(50)-2-1	(50)-5	(50)-2-1	(50)-5	(50)-2-1	(50)-1-1
Ots4	(56)-2-2	(54)-2-2	(57)-2-2	(54)-5	(54)-5	(48)-2-1
Ots5	(45)-2-2	(45)-5	(45)-5	(45)-5	(45)-2-2	(45)-5
Ots6	(57)-1-3	(57)-1-3	(57)-5	(57)-5	(57)-5	(57)-5
PuPuPy	(53)-5	(53)-5	(52)-4-5	(53)-5	(53)-5	(53)-5
Sfo8	(60)-2-4	(60)-2-4	(60)-2-4	(55)-2-4	(55)-2-4	(60)-2-3
Sfo12	(50)-5	(50)-5	(50)-5	(50)-5	(50)-5	(50)-5
Sfo18	(52)-5	(52)-5	(52)-5	(52)-5	(52)-5	(52)-5
Sfo23	(52)-5	(52)-5	(52)-5	(52)-5	(52)-5	(52)-5
Ssa4	(57)-2-2	(57)-2-2	(57)-2-2	(57)-3-4	(57)-5	(57)-2-2
Ssa14	(52)-1-2,3	(52)-1-2	(52)-1-2	(52)-1-2	(52)-1-2	(52)-1-2
Ssa85	(58)-2-2	(60)-5	(58)-2-2	(57)-3-3	(57)-3-3,4	(58)-2-1
Ssa171	(56)-1-1	(56)-1-1	(56)-1-1	(57)-5	(57)-5	(56)-1-3,4
Ssa197	(57)-1-3,4	(57)-1-1	(57)-4-1	(57)-1-2	(57)-1-2	(57)-1-2
Ssa202	(58)-5	(58)-5	(58)-5	(58)-5	(58)-5	(58)-5
Ssa289	(46)-5	(46)-5	(46)-5	(46)-5	(46)-5	(46)-5
Ssa293	(53)-2-1	(53)-2-1	(53)-2-1	(53)-2-3	(53)-2-2	(53)-2-1,2
μSat15	(57)-5	(57)-5	(57)-5	(57)-5	(57)-5	(57)-5
μSat60	(60)-2-2	(60)-3-1	(60)-2-2	(57)-2-1	(57)-3-3	(60)-2-2
μSat73	(57)-2-2	(57)-2-2	(57)-3-2	(57)-2-2	(57)-2-1,2	(57)-5

[a] Code for product quality (after Pepin et al. 1995): 1) amplification of one or two bands and no stutter, 2) amplification of one or two bands and some stutter, 3) multiple bands and no smearing, 4) multiple bands and smearing, 5) no amplification at all.
[b] Code for allelic size range: 1) 60-120 b, 2) 120-180 b, 3) 180-240 b, 4) 240-300 b, 5) >300 b.

Table 2. Summary of PCR product quality by species for all microsatellite loci screened.

Species	No. Loci Screened	Score 1-2	% Score 1-2	Score 3-4	No Product
Chinook	35	22	63%	2	11
Coho	35	19	54%	4	12
Sockeye	35	18	51%	5	12
Pink	35	12	34%	4	19
Chum	35	11	31%	6	18
Atlantic	35	18	51%	3	14

Appendix E. SINE and Transposon Sequences Generate High-Resolution DNA Fingerprints: Screening for Genetic Damage in Pink Salmon (*Oncorhynchus gorbuscha*) Exposed to Prudhoe Bay Crude Oil

Ben Allen Greene[1, 2*]
James E. Seeb[1]

[1]Alaska State Department of Fish and Game, Genetics laboratory
333 Raspberry Road
Anchorage, Alaska 99518-1599

[2]Institute of Arctic Biology, University of Alaska, Fairbanks.

[*]Corresponding Author: FAX (907) 267-2435; E-mail: BENG@FISHGAME.STATE.AK.US

Running Title: DNA fingerprinting of pink salmon.
Keywords: *Oncorhynchus gorbuscha*, PCR, SINE, *Tc1*, DNA fingerprint, pink salmon, genotoxicology

ABSTRACT.

We report the development and application of a technique to obtain high resolution fluorescent DNA fingerprints from individual pink salmon (*Oncorhynchus gorbuscha*). A dye-labeled primer complementary to the *Tc1*-like transposon together with an unlabeled primer for the *SmaI* SINE repeat motif are used to simultaneously amplify between 50 and 70 independent loci ranging in size from 60 to 535 bases using the polymerase chain reaction (PCR). These fluorescently labeled DNAs are separated on a denaturing acrylamide gel and resolved using an automated DNA sequencing instrument. We find the complex pattern of amplified fragments to be a reliable, specific fingerprint for the individual. Here we show faithful mendelian inheritance of these loci within families of single pair matings. Furthermore, we use these families and this technology to test for genetic lesions induced by exposure of pink salmon embryos to Alaska North Slope crude oil. Embryos representing full siblings of eight different families were incubated in contact with either oiled gravel or clean gravel (control) following fertilization and allowed to develop at 5.6°C for 55 to 67 days, depending on the family, until eye pigment was visible through chorion. Whereas significantly reduced embryo survivorship and a higher incidence of abnormal embryo phenotypes were observed for the oiled embryos, specific mutations (loss or gain of alleles on DNA fingerprints) were not observed in comparing 195 oiled and 187 control embryo fingerprints. These results indicate that following polycyclic aromatic hydrocarbon (PAH) exposure, overall chromosomal integrity is maintained without significant insertion/deletions, although single nucleotide substitutions cannot be detected using this technique. Despite negative results detecting PAH-associated genetic lesions, this DNA fingerprinting technology promises to be a powerful tool with many population and molecular genetic applications both for salmonids and other species.

Introduction.

Field studies following the 1989 *Exxon Valdez* oil spill revealed that some populations of pink salmon (*Oncorhynchus gorbuscha*) may have suffered heritable genetic damage (Bue et al., 1996). Unfortunately existing techniques for mutation detection are generally limited to screening specific, preselected genomic targets. In this paper we report a novel technique to fingerprint genomes and apply this technique to screen for genotoxic mutations in pink salmon exposed to crude oil. It has only recently become possible to obtain reproducible, high resolution DNA fingerprints and unambiguous genotypes from large numbers of individuals. This technology is revolutionizing the fields of population and behavioral genetics, studies of molecular evolution, and forensics (Burke et al., 1991; Lynch, 1991). Molecular genotyping has relied heavily on RFLPs (restriction fragment length polymorphisms) and PCR amplified mini- and microsatellite loci. Microsatellites (also called VNTRs referring to the variable number of tandem repeats of a 2-5 nucleotide "core") are found scattered throughout the genome and have been particularly informative as they are among the most mutable sequences known (Burke et al., 1991; Beckmann and Weber, 1992). Nonetheless, application of this technique has been limited by the difficulty of isolating useful VNTR primers, a task which generally requires cloning and is time and labor intensive. The use of random amplified polymorphic DNA (RAPD) markers, on the other hand, is technically simple, rapid and inexpensive, thus amenable to studies requiring high throughput, however, serious questions of reproducibility and difficulty in demonstrating heritability of loci generated with low stringency PCR remain unsolved (Lynch and Milligan, 1994). Here we report a technique generating high-resolution, high-stringency DNA fingerprints using two families of repetitive genomic DNA sequences for PCR: short interspersed repetitive elements (SINEs) and transposons.

The first SINE to be well characterized was the human *Alu* family (reviewed by Novick et al., 1996). These elements comprise roughly 5% of the human genome with an estimated 500,000 dispersed copies. On average there is an *Alu* element every 5 kb. Similar highly repetitive sequences (the *SmaI* family, for example) have been found in the genus *Oncorhynchus* (Okada, 1991). These "molecular benchmarks" are thought to be evolutionarily stable and present in all members of a species.

Mobile genetic elements, or transposons, are another ubiquitous component of eukaryotic genomes. Transposons have become a valuable tool in molecular genetic studies of many organisms from maize to drosophila (Berg and Howe, 1989). One highly conserved family of elements, the *Tc1* elements, have been found in diverse phyla including fish (Radice et al., 1994). Characterization of *Tc1*-like elements in various fish species revealed that these elements appear to be no longer mobile, hence, like the *SmaI* elements, are probably stable evolutionary artifacts. There are roughly 50,000 copies of *Tc1* in pink salmon (Goodier and Davidson, 1994). We reasoned that both *SmaI* and *Tc1* would be useful chromosomal landmarks for mapping studies, for stock identification and could also be used to efficiently screen for chromosomal aberrations and mutations caused by exposure of salmon embryos to crude oil. This study is also designed to "fill in the middle ground", as it is coordinated with flow cytometric analyses (Miller et al., 1994; Habicht et al, appendix A; this report.) of whole chromosomes from oiled and non-oiled sibling embryos to detect macrolesions as well as with fine-scale comparative sequencing efforts designed to detect single nucleotide changes in selected loci.

With well over 10^9 basepairs in most higher eukaryote genomes, comparative sequencing as an assay to detect mutations can only be used to screen, at best, an infinitesimally small portion of a genome. In the field of genotoxicology the target(s) of mutation are typically

unknown, so it is not obvious which specific, characterized genes should be preselected for sequencing. On the other hand, flow cytometric and cytological studies can only yield roughly megabase resolution for detecting broken chromosomes and macrolesions. Few techniques exist which offer the power to detect intermediate scale genetic lesions (between nucleotide substitution and chromosome arm breakage) associated with exposure to a suspected mutagen. The present DNA fingerprinting approach has the advantages that no a priori selection of target loci is made, yet one can perform high throughput screens for mutations, rearrangements or insertions/deletions as small as 2 base pairs in many loci dispersed throughout the genome. Since some 70 loci, varying in size from 50 to 535 bp are screened simultaneously per sample, we can, in effect, screen over 20,000 bases of DNA per lane or well over 500,000 bases per gel, making this technique a much more efficient screen for many types of mutations than is comparative sequencing.

Materials and Methods.

Seventy-five ng of template DNA isolated from parents and progeny of pink salmon from controlled single-pair hatchery matings were PCR amplified in 25 *ul* with 200*uM* each dNTP, 10mM Tris, pH 8.3, 50 mM KCl, 7.5 pmol Hex dye-labeled *Tc1* primer (5' gTA TgT AAA CTT CTg ACC CAC Tgg 3', sequence courtesy of Dr. Zoltan Ivics, University of MN, St. Paul) and 7.5 pmol *SmaI* (unlabeled) primer (5' TAC CAA CTg AgC TAC AgA Agg ACC 3'; Kido et al., 1991), 2.0 mM $MgCl_2$, and 2U Amplitaq DNA polymerase (Perkin Elmer, Foster City, CA.) using a Perkin Elmer 9600 thermalcycler. An initial denaturation for 30 seconds at 96°C was followed by 35 cycles of (94°, 15s, 62°, 20s; 72°, 150s) with a final extension at 72° for 2 hours. PCR products were diluted to 100 *ul* with dH_2O, and 4 *ul* of this were dried, mixed with internal lane standard (Tamara 2500, Applied Biosystems Incorporated (ABI), Foster City, CA.), denatured, and loaded onto a 5.25% Acrylamide, 6M Urea denaturing gel which was run using an ABI 377 automated DNA sequencer according to the manufactures instructions. Lane-to-lane comparisons of the population of fluorescently labeled amplified loci was analyzed using Genescan and Genotyper software (ABI, Foster City, CA.).

Results.

Tc1/ *SmaI* fingerprints from 195 and 187 individual pink salmon embryos incubated in contact with oiled and clean gravel, respectively, and representing full siblings from eight families of single pair matings were compared along with the DNA fingerprints from parental tissue. Figure 1 shows a representative segment of the aligned electropherograms from adjacent lanes of a DNA fingerprinting gel for family 7B. Within this segment, spanning bases 260-360 we resolved seven loci common to all members of the family (small arrowheads at bottom) and 5 loci which were polymorphic within the family (large arrows at top). We resolved roughly 60-70 loci per individual for an entire gel, and summaries of the allelic distribution for these loci are found in Tables 1-8. Part A summarizes the numbers of total fragments (loci) scored as being present or absent for each family. Details of the polymorphic loci are tabulated in part B. Several features are evident. Many common as well as many polymorphic amplified fragments (alleles) are detected for each individual. All loci appear to be transmitted in a mendelian fashion. Polymorphic fragments segregating in progeny can always be identified within one or the other parent and no fragments are found within progeny which are not present in a parent. Similarly, all loci which are found in the parental fingerprint are passed on and comprise part of the fingerprints for at least some of the progeny. Further note that there were no significant

differences in the segregation of polymorphic loci in oiled versus control sibling progeny.for the eight families compared. Finally, it is important to note that families are associated with several unique loci. For example, both of the segregating loci migrating at 88.7 and 91.7 bases were found in family 3B and not seen in any of the other seven families; likewise a segregating fragment at 191.9 was unique to family 4B. Conversely, some loci (i.e. at 116/117 nt) were found segregating in most of the families analyzed.

Direct comparisons between oiled and control progeny fails to detect any gain or loss of alleles or aberrant segregation, although our sample sizes were probably not sufficiently large to detect aberrant segregation ratios.

Discussion.

We developed a powerful technique to fingerprint pink salmon genomes. Resolving the array of loci amplified using fluorescently labeled *Tc1* and unlabeled *SmaI* primers provides a highly detailed "bar code" which, in our hands, has been used successfully to identify members of a family and resolve mixed samples. That *Tc1/SmaI* fingerprinting has the power to resolve families and ascertain parentage is illustrated in the following test. The experimental design for our laboratory oilings included 10 incubators with fertilized eggs of single pair matings incubating in contact with either oiled or clean gravel. Progeny of different families were contained in separate incubators or separated in a single incubator by a screen. When DNA sequence data revealed a surprising polymorphism in the cytochrome-b gene of several individuals of family 2B (data not shown), we used *Tc1/SmaI* fingerprinting to ascertain parentage. We compared *Tc1/SmaI* fingerprints of these individuals versus parental DNAs versus progeny of family 2A, from the same incubator, separated by a screen. Six amplified products appeared in fingerprints of the progeny in question which were not found in either of their presumed parents or other progeny of family 2B indicating that, indeed, these embryos were contaminants. Other parental DNAs from our matings were tested for the presence of these six loci. Of the population of 20 parents only one set of parents (2A) had all six loci indicating that the embryos in question were from eggs which had fallen down from above the screen and were members of family 2A, not 2B. The frequency that at least one parent of each of the 10 parental pairs contained each of the six diagnostic loci were as follows: 0.5; 0.2; 0.1; 0.5; 0.1; 0.3. Hence the probability, within our parental population, of having all six loci is 1.5×10^{-4}, or 1 in 6667. *Tc1/SmaI* fingerprinting has proved a very valuable tool in ascertaining parentage and serves as a vital control for our comparative sequencing work.

Loci amplified using this technique follow strict Mendelian inheritance, consistent with other studies of SINE repeat sequences in salmon (Spruell & Thorgaard, 1996). Application of this technique to mutation studies should provide a new tool in detecting many types of lesion. The resolving power using the automated sequencer is +/- 1-3 bases for most amplified products; thus even small insertions, deletions and many chromosomal rearrangements should be readily detectable. Several human cancers are associated with the expansion of di- or trinucleotide repeats at specific loci (Bissler et al., 1994). *Tc1/SmaI* fingerprinting has the resolving power to score novel microsatellite alleles without requiring a priori sequence information of the target locus. In fact the pattern of amplified fragments, with diagnostic "shadow bands", indicates that many of the loci amplified using *Tc1/SmaI* include nucleotide repeats (Ginot et al., 1996).

PCR amplification with various SINE primers also revealed that the 5' and 3' ends of a given SINE motif are quite distinct; priming from one end typically yields many more products than priming from the other end (data not shown). This result is consistent with the results of

Spruell and Thorgaard (1996) and indicates that the structure of salmonid SINE elements may be complex.

In a carefully controlled study of between-sibling genotoxic effects of embryo incubation/development in contact with Alaska North Slope crude oil, we detected no gain or loss of alleles despite the fact that exposure to crude oil significantly (P=0.012) increased embryo mortality (average survival rate for 9 families of control embryos= 23.04%; average survival of 17.04% for siblings of the same 9 families incubated in oiled gravel) as well as inducing aberrant phenotypes (15.9% of the oiled embryos were abnormal versus only 0.19% abnormal embryos from control incubators; P=0.016; for complete data of these experiments see Habicht et al, appendix A; this report.; Seeb et al, materials and methods). Despite the damage caused by exposure to crude oil, the effects appear not to be correlated with significant nucleotide insertion/deletion events, nor are they associated with grotesque chromosomal rearrangements (Habicht et al, appendix A; this report.). We conclude that North Slope crude oil does not dramatically disrupt chromosomal architecture in pink salmon, although the present results cannot be used to detect nucleotide substitution frequencies.

The utility of *Tc1/SmaI* fingerprinting for mutation screening is limited to scoring the presence or absence of segments of the genome which are amplified between adjacent *SmaI* and *Tc1* sequences. In other words, *Tc1/SmaI* can be used to inventory overall genomic integrity, but is not able to detect single base substitutions unless it occurs within the primer binding sites. Interpreting *Tc1/SmaI* fingerprints is complicated in regions where comigrating amplified PCR products exist. As *Tc1/SmaI* fingerprinting can only distinguish loci according to size, it is reasonable to estimate that some "loci" actually represent pools of uniformly sized amplified DNA fragments arising from several distinct genomic locations. Certainly, with many amplified fragments per lane, it is often difficult or impossible to discriminate between signals of similarly sized DNA fragments. Such "loci" would be expected to segregate as duplicated genes. Indeed, because the relative differences in migration for similar sized short DNAs is particularly subtle, we have seen a trend whereby segregation ratios higher than 50% are particularly common for loci defined by shorter PCR products indicating that two or more similarly sized products are being scored.

Dubrova et al. (1996) investigated increased mutation rates of humans exposed to radioactive contamination from the Chernobyl nuclear accident by hybridizing southern blots containing DNA from parents and children born in heavily contaminated areas with the multilocus 33.15 minisatellite probe and with several single-locus minisatellite probes. Mutations were identified as novel fragments present in the offspring not found in either parent. Several features of this study can be compared to the present *Tc1/SmaI* fingerprinting screen. Both *Tc1/SmaI* and minisatellite fingerprints provide individual-specific molecular genotypes with little or no information of the exact loci represented. Dubrova et al. were able to score 18 bands (loci) using probe 33.15; whereas 50-80 bands can be scored using the ABI 377 automated sequencer for *Tc1/SmaI* fingerprinting. The resolution of fragments on minisatellite blots is plus/ minus 200-300 nucleotides, whereas resolution with *Tc1*/Sma is +/- 2-3 bp. There is also intrinsic difficulty in scoring presence/absence of bands on the southern blots as hybridization yields smears in addition to discrete bands. Finally, Dubrova et al. were able to score a total of roughly 1,500 bands on southern blots of about 80 individuals each from exposed and control groups using the 33.15 probe. Using a technique like *Tc1/SmaI* for 80 individuals would allow scoring some 4000 loci, and with much higher resolution.

Further studies are needed in order to test the resolving power of *Tc1/Sma1* as an additional tool to identify structure among salmonid populations. We suspect that with some optimization the technique should be applicable to dissect relatedness of populations where the differences are too small to yield allozyme or mtDNA-detectable differentiation. An attractive feature of *Tc1/ Sma1* fingerprinting is the ability to adjust the complexity of data by altering the specificity of the PCR reactions. Many different salmonid SINE elements have been characterized (Kido et al, 1991), each with distinct species abundance and relative copy numbers, providing many choices for obtaining fingerprints with an optimal display of data for many different applications.

In conclusion, we feel that we have a powerful new tool for population and molecular genetics. Further studies with larger sample sizes are needed to ascertain whether we can detect genotoxic effects of exposure to PAHs, and to determine the resolving power of this technique for stock identification.

Literature cited.

Allendorf, FW Thorgaard GH. 1984. Tetraploidy and the Evolution of Salmonid Fishes. Evolutionary Genetics of Fishes; ed. BJ Turner (Plenum Publishing).

Baker, R.J., Van Den Bussche, R.A., Wright, A.J., Wiggens, L.E., Hamliton, M.J., Reat, E.P., Smith, M.H., Lomakin, M.D., and Chesser, R.K. 1996. High levels of genetic change in rodents of Chernobyl. Nature. 380:707-708.

Beckmann, JS Weber JL. Survey of Human and Rat Microsatellites. Genomics. 1992; 12:627-631.

Berg, DE Howe MM. Mobile DNA. American Society for Microbiology. 1989; (Washington, DC).

Bernatchez, L., and Osinov, A. 1995. Genetic diversity of trout (genus Salmo) from its most eastern native range based on mitochondrial DNA and nuclear gene variation. Mol. Ecol. 4:285-297.

Bickham J.W. 1990. Flow cytometry as a technique to monitor the effects of environmental genotoxins on wildlife populations. In: Sandhu S.S., W.R. Lower, F.J. de Serres, W.A. Suk, R.R. Tice (eds) In situ evaluations of biological hazards of environmental pollutants. Plenum Press, New York, pp 97-108.

Bissler, JJ Cicardi M Donaldson VH Gatenby PA Rosen FS. A Cluster of Mutations within a short Triplet Repeat in the C1 Inhibitor Gene. Proc. Natl. Acad. Sci. USA. 1994; 91:9622-9625.

Bue, B.G. 1996 Effects of the *Exxon Valdez* oil spill on pink salmon embryos and preemergent fry. Am. Fisher. Soc. Symp. 18:000-000.

Burke, T Hanotte O Bruford MW Cairns E. Multilocus and Single Locus Minisatellite Analysis in Population Biological Studies. in: DNA Fingerprinting: Approaches And Applications. 1991; ed. by T. Burke, G. Dolf, AJ Jeffreys & R. Wolf (Birkhauser Verlag Basel/ Switzerland).

DiGiovanni, J., Beltran, L., Rupp, A., Harvey, R.G., Gill, R.D. 1993. Further analysis of c-Ha-ras mutations in papillomas initiated by several polycyclic aromatic hydrocarbons and papillomas from uninitiated, promoter-treated skin in SENCAR mice. Mol. Carcinogen. 8:272-279.

Dubrova, Y.E., Nesterov, D.N., Krouchinsky, N.G., Ostapinko, V.A., Neumann, R., Neil, D.L., Jeffreys, A.J. 1996. Human minisatellite mutation rate after the Chernobyl accident. Nature 380:683-686.

Fong, A. T., R. H. Dashwood, R. Cheng, C. Mathews, B. Ford, J. D. Hendricks, and G. S. Bailey. 1993. Carcinogenicity, metabolism and ki-ras proto-oncogene activation by 7,12-dimethylbenz[a]anthracene in rainbow trout embryos. Carcinogenesis 14:629-635.

Geiger, H.J., B.G. Bue, S.Sharr, A.C.Wertheimer, and T.M.Willette. *in press*. A life history approach to estimating damage to Prince William Sound Pink Salmon from the *Exxon Valdez* oil spill. In Rice, S.D., R.B. Spies, D.A. Wolfe, and B.A. Wright [eds.] Exxon Valdez Oil Spill Symposium Proceedings. Am. Fish. Soc. Symp. 18:000-000.

Ginot, F Bordelais I Nguyen S Gyapay G. Correction of some Genotyping Errors in Automated Fluorescent Microsatellite Analysis by Enzymatic Removal of one Base Overhangs. Nucleic Acids Res. 1996; 24:540-541.

Goodier, JL Davidson WS. Tc1 Transposon-like Sequences are Widely Distributed in Salmonids. J. Mol. Bio. 1994; 241:26-34.

Habicht, C., Miller, G.D., Evans, D., Heintz, R., Seeb, J.E. 1996. Intensive flow cytometry analyses do not detect macrolesions in pink salmon embryos following crude oil exposure. Appendix A; This report.

Kido, Y Aono M Yamaki T Matsumoto K-I Murata S Saneyoshi M Okada N. Shaping and Reshaping of Salmonid Genomes by Amplification of tRNA-Derived Retroposons During Evolution. Proc. Natl. Acad. Sci. USA. 1991; (88):2326-2330.

Lynch, M. Analysis of Population Genetic Structure by DNA Fingerprinting. in: DNA Fingerprinting: Approaches And Applications. 1991; ed. by T. Burke, G. Dolf, AJ Jeffreys, & R. Wolf(Birkhauser Verlag Basel/Switzerland).

Lynch, M BG Milligan. Analysis of Population Genetic Structure with RAPD Markers. Molecular Ecology. 1994; 3:91-99.

McBee, K., and J.W. Bickham. 1988. Petrochemical-related DNA damage in wild rodents detected by flow cytometry. Bull Environ Contam Toxicol 40:343-349.

Miller, G.D., Seeb, J.E., Bue, B.G., Sharr, S. 1994. Saltwater exposure at fertilization induces ploidy alterations, including mosaicism, in salmonids. Can. J. Fish. Aquat. Sci. 51:42-49.

Moles, A., M.M. Babcock, and S.D. Rice. 1987. Effects of oil exposure on pink salmon, *O. gorbuscha*, alevins in a simulated Intertidal Environment. Marine Environmental Research 21:49-58.

Novick, GE Batzer MA Deininger PL Herrera RJ. The Mobile Genetic Element Alu in the Human Genome. BioScience. 1996; 46:32-41.

Okada, N. SINEs: Short Interspersed Repeated Elements of the Eukaryotic Genome. Trend Ecol. Evol. 1991; 6:358-361.

Radice, AD Bugaj B Fitch DHA Emmons SW. Widespread Occurrence of the Tc1 Transposon Family: Tc1-like Transposons from Teleost Fish. Mol. Gen. Genet. 1994; 244:606-612

Allendorf, F.W., G.H. Thorgaard. 1984. Tetraploidy and the evolution of salmonid fishes. In: B.J. Turner (ed) Evolutionary genetics of fishes. Plenum Press, New York, pp 1-53.

Rice, S.D., D.A. Moles, and J.W. Short. 1975. The effect of Prudhoe Bay crude oil on survival and growth of eggs, alevins, and fry of pink salmon, *Oncorhynchus gorbuscha*. Pages 503-507 *in* Proceedings of the 1975 Conference on Prevention and Control of Oil Pollution, San Francisco, Ca. American Petroleum Institute. Washington, D.C.

Sharr, S., B.G. Bue, S. D. Moffitt, and A.K. Craig. 1994a. Injury to salmon eggs and preemergent fry in Prince William Sound - F/S 1. State/Federal Natural Resources Damage Assessment Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, Ak.

Sharr, S., J.E. Seeb, B.G. Bue, S. D. Moffitt, A.K. Craig, and G.D. Miller. 1994b. Injury to salmon eggs and preemergent fry in Prince William Sound - R60C. State/Federal Natural Resources Restoration Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, Ak.

Sharr, S., J.E. Seeb, B.G. Bue, S. D. Moffitt, A.K. Craig, and G.D. Miller. 1994c. Injury to salmon eggs and preemergent fry in Prince William Sound - R93003. State/Federal Natural Resources Restoration Final Report. Alaska Department of Fish and Game, Division of Commercial Fisheries Management and Development. Cordova, Ak.

Spruell, P., Thorgaard, G.H. 1996. SINE sequences detect DNA fingerprints in salmonid fishes. Heredity. 76:317-324.

Wright, J. M. and P. Bentzen. 1994. Microsatellites: genetics markers of the future. Reviews in Fish Biology and Fisheries 4:384-388.

Acknowledgements.

This work was funded by the Exxon Valdez Oil Spill Trustee Council progect 11961911, and the Alaska Department of Fish and Game Division of Commercial Fisheries Managment and Development. We would like to thank Dr. Zoltan Ivics, University of MN, St. Paul for the *Tc1* primer sequence, Dr. Norihiro Okada for advice, Eric Kretchmer for technical assistance and Chris Habicht for assistance

Figure 1. Representative segment of aligned fingerprint electropherograms from 2 parental and 8 progeny DNAs of family 7B showing amplified loci in the range of 245-345 bases in length. The first two fingerprints, (#739 and 750) were from paternal and maternal DNAs, respectively. The next 4 individuals (#1134, 1135, 1139, and 1140) were progeny incubated in oil, whereas the final 4 siblings (# 1124, 1125, 1127, 1128) were sibling progeny taken from a control incubator. Of the 13 scorable loci in this segment, seven loci were common to all members of the family (small arrowheads at bottom) and 5 loci were polymorphic within the family (large arrows at top). For example, two fragments at 262 and 343 bases segregated in both progeny and parentals, whereas the fragments at 246 and 319 bases were present in all members.

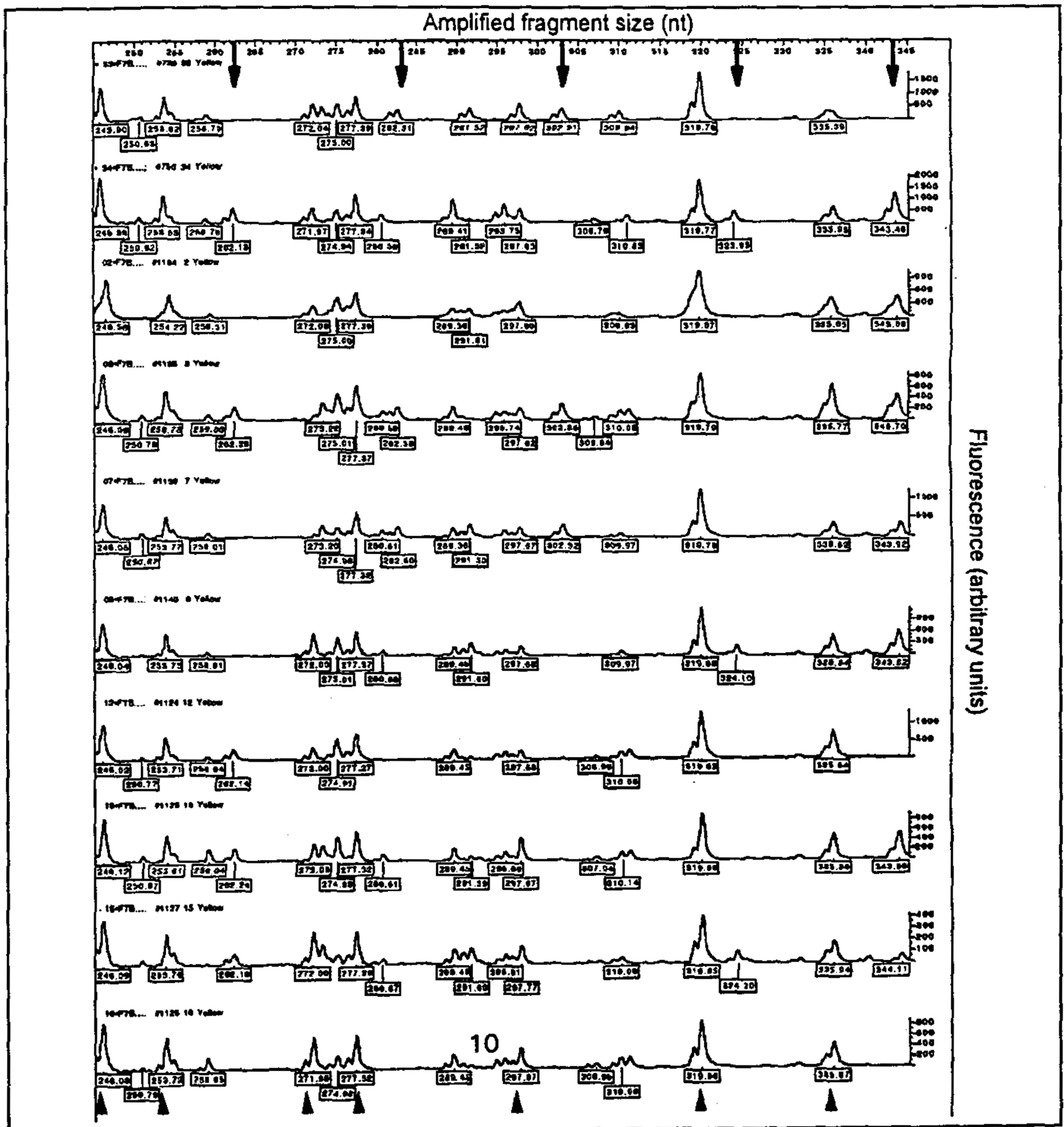


Table 1. Allelic distribution of all loci amplified and scored from family 2B.

<u>(A) Summary of all loci scored</u>

Number of control progeny screened	34
Number of oiled progeny screened	25
Total number of loci scored from each individual	70
Number of loci common to all members	55 (79%)
Loci segregating in both parents and progeny	14 (20%)
Loci segregating in progeny; present in both parents	1 (1%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	117	126	128	204	208	218	283	310	324	343	394	402	406	435	524
Sire 2B	0	0	0	1	0	1	1	1	0	0	0	0	1	1	1
Dam 2B	1	1	1	0	1	0	0	0	1	1	1	1	1	0	0
# of total progeny	33	32	30	47	29	32	39	44	40	37	30	34	41	33	26
% of total progeny	0.56	0.54	0.51	0.80	0.49	0.54	0.66	0.75	0.68	0.63	0.51	0.58	0.69	0.56	0.44
# of control progeny	20	19	16	27	16	19	21	26	22	22	16	21	23	18	15
% of control progeny	0.59	0.56	0.47	0.62	0.47	0.56	0.62	0.76	0.65	0.65	0.47	0.62	0.68	0.53	0.44
# of oiled progeny	13	13	14	20	13	13	18	18	18	15	14	13	18	15	11
% of oiled progeny	0.52	0.52	0.56	0.80	0.52	0.52	0.72	0.72	0.72	0.60	0.56	0.52	0.72	0.60	0.44

Table 2. Allelic distribution of all loci amplified and scored from family 3B.

(A) Summary of all loci scored

Number of control progeny screened	28
Number of oiled progeny screened	27
Total number of loci scored from each individual	72
Number of loci common to all members	53 (74%)
Loci segregating in both parents and progeny	12 (17%)
Loci segregating in progeny; present in both parents	7 (10%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	88.7	91.7	116	128	161	204	208	259	272	303	307	310	323	343.8	270	402	433	435	452
Sire 3B	1	0	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	0	0
Dam 3B	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1
# of total progeny	33	30	32	32	29	47	39	44	40	37	30	34	30	33	26	30	29	29	26
% of total progeny	0.60	0.55	0.58	0.58	0.53	0.85	0.71	0.80	0.73	0.67	0.55	0.62	0.55	0.60	0.47	0.55	0.53	0.53	0.47
# of control progeny	15	16	13	18	14	25	20	22	20	20	16	18	15	18	12	15	15	16	11
% of control progeny	0.54	0.57	0.46	0.64	0.50	0.89	0.71	0.79	0.71	0.71	0.57	0.64	0.54	0.64	0.43	0.54	0.54	0.57	0.39
# of oiled progeny	18	14	19	14	15	22	19	22	20	17	14	16	15	15	14	15	14	13	15
% of oiled progeny	0.67	0.52	0.70	0.52	0.56	0.81	0.70	0.81	0.74	0.63	0.52	0.59	0.56	0.56	0.52	0.56	0.52	0.48	0.56

Table 3. Allelic distribution of all loci amplified and scored from family 4B.

<u>(A) Summary of all loci scored</u>

Number of control progeny screened	26
Number of oiled progeny screened	26
Total number of loci scored from each individual	75
Number of loci common to all members	63 (84%)
Loci segregating in both parents and progeny	10 (13%)
Loci segregating in progeny; present in both parents	2 (3%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	106	116	177	192	259	266	272	288	310	324	344	398
Sire 4B	1	1	1	1	1	1	0	1	1	1	1	1
Dam 4B	0	0	0	1	1	0	1	0	0	0	0	0
# of total progeny	29	32	27	33	38	28	32	30	25	24	30	27
% of total progeny	0.56	0.62	0.52	0.63	0.73	0.54	0.62	0.58	0.48	0.46	0.58	0.52
# of control progeny	16	15	13	14	19	11	15	17	12	12	16	14
% of control progeny	0.62	0.58	0.50	0.54	0.73	0.42	0.58	0.65	0.46	0.46	0.62	0.54
# of oiled progeny	13	17	14	19	19	17	17	13	13	12	14	13
% of oiled progeny	0.50	0.65	0.54	0.73	0.73	0.65	0.65	0.50	0.50	0.46	0.54	0.50

Table 4. Allelic distribution of all loci amplified and scored from family 5B.

<u>(A) Summary of all loci scored</u>

Number of control progeny screened	19
Number of oiled progeny screened	22
Total number of loci scored from each individual	75
Number of loci common to all members	64 (85%)
Loci segregating in both parents and progeny	9 (12%)
Loci segregating in progeny; present in both parents	1 (3%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	90	127	182	251	287	298	307	324	344	368
Sire 5B	0	1	1	1	0	1	0	1	0	1
Dam 5B	1	1	0	0	1	0	1	0	1	0
# of total progeny	25	32	22	24	19	22	22	21	22	19
% of total progeny	0.61	0.78	0.54	0.59	0.46	0.54	0.54	0.51	0.54	0.46
# of control progeny	12	15	8	12	9	10	8	10	9	8
% of control progeny	0.63	0.79	0.42	0.63	0.47	0.53	0.42	0.53	0.47	0.42
# of oiled progeny	13	17	14	12	10	12	14	11	13	11
% of oiled progeny	0.59	0.77	0.64	0.55	0.45	0.55	0.64	0.50	0.59	0.50

Table 5. Allelic distribution of all loci amplified and scored from family 7B.

(A) Summary of all loci scored

Number of control progeny screened	20
Number of oiled progeny screened	20
Total number of loci scored from each individual	75
Number of loci common to all members	62 (83%)
Loci segregating in both parents and progeny	12 (16%)
Loci segregating in progeny; present in both parents	1 (1%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	127	208	226	259	262	283	303	324	332	344	408	493	524
Sire 7B	1	0	0	1	0	1	1	0	1	0	0	0	0
Dam 7B	0	1	1	1	1	0	0	1	0	1	1	1	1
# of total progeny	25	21	22	31	21	19	19	20	19	24	18	22	22
% of total progeny	0.63	0.53	0.55	0.78	0.53	0.48	0.48	0.50	0.48	0.60	0.45	0.55	0.55
# of control progeny	12	11	11	17	9	10	10	10	8	11	9	14	9
% of control progeny	0.60	0.55	0.55	0.85	0.45	0.50	0.50	0.50	0.40	0.55	0.45	0.70	0.45
# of oiled progeny	13	10	11	14	12	9	9	10	11	13	9	8	13
% of oiled progeny	0.65	0.50	0.55	0.70	0.60	0.45	0.45	0.50	0.55	0.65	0.45	0.40	0.65

Table 6. Allelic distribution of all loci amplified and scored from family 8B.

<u>(A) Summary of all loci scored</u>

Number of control progeny screened	26
Number of oiled progeny screened	30
Total number of loci scored from each individual	68
Number of loci common to all members	60 (88%)
Loci segregating in both parents and progeny	6 (9%)
Loci segregating in progeny; present in both parents	2 (3%)

(B) Inheritance of polymorphic loci: *size of amplified fragment* (allele) in nucleotides.

	153	156	177	250	272	283	286	303
Sire 8B	1	1	1	1	1	1	0	0
Dam 8B	0	1	0	1	0	0	1	1
# of total progeny	33	40	30	41	30	30	28	32
% of total progeny	0.59	0.71	0.54	0.73	0.54	0.54	0.50	0.57
# of control progeny	17	15	12	19	13	10	12	15
% of control progeny	0.65	0.58	0.46	0.73	0.50	0.38	0.46	0.58
# of oiled progeny	16	25	18	22	17	20	16	17
% of oiled progeny	0.53	0.83	0.60	0.73	0.57	0.67	0.53	0.57

Table 7. Allelic distribution of all loci amplified and scored from family 10B.

(A) Summary of all loci scored	
Number of control progeny screened	19
Number of oiled progeny screened	18
Total number of loci scored from each individual	73
Number of loci common to all members	63 (86%)
Loci segregating in both parents and progeny	12 (16%)
Loci segregating in progeny; present in both parents	1 (1%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	118	141	173	218	251	283	303	315	324	332
Sire 10B	0	0	0	1	1	1	1	0	0	1
Dam 10B	1	1	1	0	1	0	0	1	1	0
# of total progeny	24	20	20	18	28	22	21	17	14	25
% of total progeny	0.65	0.54	0.54	0.49	0.76	0.59	0.57	0.46	0.38	0.68
# of control progeny	13	11	10	10	15	11	11	8	8	13
% of control progeny	0.68	0.58	0.53	0.53	0.79	0.58	0.58	0.42	0.42	0.68
# of oiled progeny	11	9	10	8	13	11	10	9	6	12
% of oiled progeny	0.61	0.50	0.56	0.44	0.72	0.61	0.56	0.50	0.33	0.67

Table 8. Allelic distribution of all loci amplified and scored from family 11B.

<u>(A) Summary of all loci scored</u>

Number of control progeny screened	23
Number of oiled progeny screened	19
Total number of loci scored from each individual	55
Number of loci common to all members	47 (85%)
Loci segregating in both parents and progeny	4 (7%)
Loci segregating in progeny; present in both parents	4 (7%)

(B) Inheritance of polymorphic loci: size of amplified fragment (allele) in nucleotides.

	85	116	128	177	249	295	328	344
Sire 11B	1	1	1	1	1	0	1	0
Dam 11B	0	1	1	1	1	1	0	1
# of total progeny	33	36	30	28	32	30	27	32
% of total progeny	0.79	0.86	0.71	0.67	0.76	0.71	0.64	0.76
# of control progeny	18	15	17	17	18	13	12	15
% of control progeny	0.78	0.65	0.74	0.74	0.78	0.57	0.52	0.65
# of oiled progeny	15	11	13	11	14	17	15	17
% of oiled progeny	0.79	0.58	0.68	0.58	0.74	0.89	0.79	0.89

Appendix F. An Evaluation Of Molecular Genetic Damage To Pink Salmon Embryos Experimentally Exposed To Weathered Prudhoe Bay Crude Oil

Nirmal K. Roy[1], Joseph Stabile[1,3], James E. Seeb[2], Christopher Habicht[2], Isaac Wirgin[1],

[1]Institute of Environmental Medicine, New York University Medicine
Long Meadow Road
Tuxedo, New York 10987
Phone:914-351-2415
Fax:914-351-5472

[2]Alaska Department of Fish and Game
333 Rasberry Road
Anchorage, Alaska 99518
Phone:907-267-2385

[3]Department of Biology, Iona College
715 North Avenue
New Rochelle, New York 10801
Phone:914-633-2253

ABSTRACT
Previous studies demonstrated reduced survivorship of pink salmon embryos from populations in Prince William Sound, AK, that were exposed to *Exxon Valdez*-released oil compared to populations from matched non-oiled streams. Survivorship was also significantly decreased in embyros from lineages that were oiled in Prince William Sound and reared in clean water under controlled hatchery conditions compared to the descendants of non-oiled lineages. This suggests that the effect of oiling on pink salmon populations was persistent and could be transmitted inter-generationally. However, the ability of environmentally-released oil to cause DNA sequence alterations in natural populations has yet to be demonstrated. We used PCR analysis to screen for alterations in the K-*ras* oncogene in DNA from pink salmon embryos that were exposed under controlled laboratory conditions to weathered Prudhoe Bay crude oil. PCR and direct DNA sequence analyses were used to identify mutational hotspots within exons 1 and 2 of K-*ras* and 3' primer mismatch analysis was used to determine the frequency of mutations in the 40 offspring of two families of pink salmon which were experimentally exposed to oiled or clean gravel. Mutations were only observed at codons 12, 13, and 61 of K-*ras*, sites which are frequently mutated in animal and human tumors. All mutations resulted in deduced amino acid substitutions. As expected, in all individuals exhibiting mutations, copy number of the normal allele exceeded that of the mutated allele. The frequencies of mutations in oiled embryos at K-*ras* exons 1 and 2 were 68% and 41%, respectively. K-*ras* mutations were not observed in siblings that were exposed to clean gravel or in the parents of the two experimental matings. These results indicate that exposure of pink salmon embryos to weathered Prudhoe Bay crude oil under controlled laboratory conditions can elicit somatic cell mutations in high frequency at mutational hotspots in genes such as K-*ras*. However, the frequency of these events in oiled

natural populations of pink salmon and other vulnerable species in Prince William Sound and the heritability of these mutations within oiled lineages has yet to be evaluated.

Key Words: K-*ras*, pink salmon, mismatch PCR assay, crude oil, and genetic damage.

INTRODUCTION

Environmental exposure of feral populations to xenobiotics can result in sublethal biological effects. These may include biochemical, immunological, teratogenic, behavioral, and reproductive impairment (Rand et al. 1995). Additionally, the prevalence of environmentally-induced diseases such as cancer is sometimes elevated in exposed populations (Hawkins et al. 1995). The etiology of environmentally-induced diseases can sometimes be attributed to xenobiotics such as polycyclic aromatic hydrocarbons (PAHs (Baumann and Harshbarger 1995). These sublethal alterations and disease endpoints, while damaging at the organismic and perhaps even for the short term at the population level, are not transmitted inter-generationally and therefore are of little consequence on an evolutionary time scale. In contrast, genetic damage can potentially be transmitted inter-generationally and therefore result in persistent alterations in population performance and eventually community structure (Anderson et al. 1994). However, few studies have documented the heritability of toxicant-induced mutations and resulting phenotypic alterations in natural populations.

Pink salmon *Oncorhynchus gorbuscha* is a commercially and recreationally important anadromous species along the Pacific coast of North America. Furthermore, pink salmon serves an important link in estuarine food chains between aquatic and terrestrial biota. Pink salmon are bi-annual spawners with fixed life expectancies of two years. Genetic studies have demonstrated significant allelic frequency differences between odd-and even-year spawners, confirming the reproductive isolation of successive year classes within individual spawning systems (Aspinwall 1974). Pink salmon spawning occurs in late summer-early fall and embryos and yolk-sac larvae incubate over-winter within the gravel substrate of both the intertidal and above the tidal zones of streams with spawning populations. In spring, post-yolk sac larvae emerge and shortly migrate to sea. Thus, young life stages of pink salmon are particularly vulnerable to xenobiotics which may persist within the benthic substrate of the intertidal zone of natal streams.

Many of the tributary streams of western Prince William Sound, AK, were oiled in March-April 1989. In 1989 and 1990, significantly increased embyro mortality was observed in pink salmon populations that were oiled in Prince William Sound compared to populations in nearby non-oiled streams (Bue et al. 1996). In 1989, this difference was observed in all intertidal areas--in 1990 mortality was only elevated within the highest intertidal zone. This observation was consistent with the greater persistence of oil residues in the upper intertidal zone. Increased embyro mortality and developmental abnormalities were also observed in Baltic herring *Clupea harengus membras* embryos that were experimentally exposed to two crude oils (Linden 1978).

Despite greatly reduced oil concentrations in all intertidal zones by the summer of 1991, embryo mortality in 1991 was even higher in oiled than in reference streams suggesting; (1) retention of petroleum residues in tissues of the 1989 year class and transmission of residues through their gametes to the 1991 year class, (2) residual oil in gravel, (3)physiological damage in adults from oil exposure as embryos resulting in lower quality gametes, (4) inherently higher mortality in

oiled streams due to factors unassociated with oil such as increased exposure to wave action, or (5) inter-generation transmission of genetic damage.

To evaluate the later hypothesis, in 1993, gametes were collected from broodstock from oiled and non-oiled streams in Prince William Sound, intrastream crosses were made, and embryos were reared under identical clean hatchery conditions (Bue et al. 1998). Despite removal for two generations from the oiling experience, embryos from oiled lineages still exhibited significantly increased mortality compared to embyros derived from non-oiled lineages. These results strongly supported a genetic etiology to peristent elevated mortality of embryos from oiled lineages. When these experiments were repeated with embryos from the 1994 year class, no difference in survivorship was observed between oiled and non-oiled lineages, a result which was consistent with a strong selective pressure against lineages with compromised embryo survivorship.

High levels of overall DNA damage using the ^{32}P postlabelling technique have been observed in natural populations of fish from highly contaminated locales which are exposed to environmental mixtures of xenobiotics, including PAHs (Stein et al. 1994; Maccubin 1994). Furthermore clastogenic alterations have been detected in fish from highly contaminated waters using karyotype, micronuclei, and flow cytometry analyses (Bickham and Smolen 1994). From example, in 1989, Pacific herring *Clupea pallasi* embryos from oiled beaches of Prince William Sound exhibited elevated levels of anaphase aberrations compared to embryos from unoiled beaches and aberration rates were highly correlated with environmental levels of aromatic hydrocarbons (Hose 1994). In contrast, highly intensive use of flow cytometry failed to detect elevated clastogenic effects in pink salmon embyros that were experimentally exposed to weathered crude oil (Habicht et al. in prep.). Approaches such as these which measure macro genetic lesions are only sensitive to overt chromosomal damage, alterations which are probably lethal and are unlikely to be transmitted inter-generationally. Thus, it is unlikely that macrolesions that may be induced by environmental mutagens are transmitted inter-generationally. However, recent advances in molecular biology now allow for sensitive screening of the genome for DNA damage that heretofore would go undetected and potentially not overtly lethal. Genes have been identified which are unusually susceptible to chemically-induced mutations and which when altered result in phenotypic change. For example, it has been demonstrated that single base alterations at critical functional domains in cellular oncogenes such as K-*ras* or tumor supressor genes such as p53 are frequent and contribute to the initiation and perhaps progression of cancer in animal and human populations (McMahon 1994).

Environmental chemicals can induce mutations by two mechanisms. PAHs are metabolized by cytochrome P4501A (CYP1A)-encoded monooxygenases to reactive electrophiles which can covalently bind to DNA forming bulky, hydrophobic DNA adducts, a result which may compromise the fidelity of DNA polymerases, destabilize the glycosidic bond between purine bases and deoxyribose and which may result in base mispairing. Although the levels of hydrophobic DNA adducts are invariably elevated in fish from highly contaminated aquatic environments (Stein et al. 1994), the ability of weathered crude oil to induce DNA lesions has not been demonstrated. In a second scenario, exposure to PAHs and metabolically resistant aromatic hydrocarbons such as PCBs, dioxins, and furans, can generate increased levels of highly reactive oxygen species including superoxide, hydrogen peroxide, and hydroxyl radicals

(Park et al. 1996), perhaps through induction of cytochrome P450s and subsequent uncoupling of electron transfer and monoxygenation (White and Stegeman 1996). Reactive oxygen species can then attack DNA causing lesions through modification of guanine and adenine bases which are then misread during replication (Kuchino et al. 1987; Olinski et al. 1995) or by decreasing levels of cytosine methylation which may increase the rate of transcription (Rhein et al. 1998). Strong evidence supporting the importance of pollution-induced reactive oxygen species in generating elevated levels of DNA damage can be found in hepatic tumors, preneoplastic lesions and normal livers of English sole *Parophrys vetulus* from contaminated sites in Puget Sound, WA (Malins et al. 1990; Malins et al. 1996).

Previous studies have indicated that mutagenic components of *Exxon Valdez* -released oil were bioavailable to fishes and other organisms residing in the subtidal and intertidal zones of tributary streams of Prince William Sound (O'Clair et al. 1996). Because PAHs are so rapidly metabolized in fish livers, it is difficult to directly measure their concentrations (Krahn et al. 1987). As an alternative, HPLC and spectrophotometry was used to quantify fluorescent metabolites of PAHs, such as benzo[a]pyrene, in fish bile. Use of this approach indicated that fish species residing in both the subtidal and intertidal zones of tributaries of Prince William Sound were exposed to elevated levels of PAHs derived from *Exxon Valdez* oil (Collier et al. 1996). Furthermore, induction of CYP1A protein and its catalyzed aryl hydrocarbon hydroxylase (AHH) enzyme activity were observed in oiled fish populations (Collier et al. 1996), including pink salmon (Weidmer et al. 1996), for up to two years after the spill indicating that these xenobiotics were bioavailable and potentially contributing to initiation of DNA damage.

Oncogenes have been implicated in the initiation of chemically induced tumors in vertebrate populations, including salmonids (Bailey et al. 1996) and other fish species (Van Beneden and Ostrander 1994). Not only are these important in signal transduction and in the initiation of the carcinogenesis, they are often sensitive to chemically-induced DNA damage. It is envisioned that DNA damage in these genes may serve as sensitive biomarkers of exposure to chemical mutagens and also as early indicators of carcinogenically relevant biological effects. Mutational activation of the K-*ras* oncogene has been described both in feral fish populations from highly impacted sites (McMahon et al. 1990; Wirgin et al. 1989) and in a chemically exposed hatchery strain of rainbow trout *Oncorhynchus mykiss* (Bailey et al. 1996). In mammals, activation of K-*ras* occurs through mutations at primarily two "hotspots"; codons 12 -13 in exon 1 and codons 59, 61-63 in exon 2. Exposure to model chemicals frequently results in "signature" mutations at specific nucleotides within sensitive codons; these mutations are often consistent with the DNA adduct spectra observed at these nucleotides in chemically treated bacteria. In winter flounder *Pleuronectes americanus* from Boston Harbor, mutations in DNA from diseased livers were observed in codons 12 and 13 of K-*ras* (McMahon et al. 1990). In Atlantic tomcod *Microgadus tomcod* from the Hudson River, mutations were observed in codon 16 of K-*ras*, a site not frequently altered in mammals (Roy and Wirgin unpublished data).

Tumors have not been observed in pink salmon populations. To our knowledge, few studies have demonstrated K-*ras* mutations in normal tissues from species that are not susceptible to neoplasia. This may have resulted from a combination of two factors; one biological and the second technical. 1) Even for sensitive genes such as K-*ras*, mutations in response to xenobiotic exposure is a relatively rare event. Thus, in normal tissues, cells with mutated alleles are rare,

and have yet to develop a selective advantage enabling them to outcompete neighboring cells with normal alleles. Thus, only a small percentage of cells in normal tissues will carry a mutated allele. In comparison, preneoplastic and neoplastic lesions contain populations of cells which are presumably clonally derived from a single or small number of mutated cells and therefore a high percentage of these cells will exhibit mutations. 2) Using standard molecular techniques, the threshold for detection of mutated alleles in a heterogeneous population of primarily normal cells is probably 5%-10%. However, sensitive PCR-based techniques have recently been developed to permit the detection of mutated alleles in a population of predominantly normal alleles. PCR-based 3'-primer mismatch assays allow for the differential amplification and detection of variant alleles with single base changes (Guo et al. 1997; Hasegawa et al. 1995). However, adoption of this approach requires prior identification of the sites and spectra of mutations at sensitive loci.

Our strategy was to initially use PCR amplification in combination with direct sequence analysis to determine if mutations were present in K-*ras* exons 1 and 2, and if so to characterize their mutational spectra. Results from these studies were then used to design PCR 3'-mismatch primers that were used in mismatch assays to sensitively determine the frequency of mutations at these sites in oiled and control embryos and their parents from two families of pink salmon.

MATERIALS AND METHODS

Embryo exposures

Incubators were designed to expose salmon embryos to oil that simulated the natural environment such that water flowed up through a column of gravel modified from the design of Marty et al. (1997). Incubators were constructed of 60-cm sections of 15-cm-diameter polyvinylchloride (PVC) pipe sealed with a PVC plate glued to one end which became the bottom. Water was admitted near the bottom and was regulated with a valve. A plate was inserted in the pipe which provided a false bottom that held gravel above the inlet hole. Water exited the incubators through a hole near its top. A polypropylene plate with 4-mm-diameter holes was used as the false bottom, rather than an aluminum plate as described by Marty et al. (1997).

River gravel with a maximum diameter of 5.1 cm was rinsed to remove fines and dried prior to oiling and loading into incubators. Treated gravel was oiled with 5.7-g oil/kg gravel using Prudhoe Bay crude oil (obtained from the National Marine Fisheries Service, Auke Bay Laboratory's supply of *Exxon Valdez* crude oil). Oil was sprayed evenly onto the gravel as it tumbled in a cement mixer. After oil was applied, gravel was laid out in one layer and exposed to sun for 4 d before being placed in the incubators. Incubators were filled with 10.8 kg of gravel. Another polypropylene plate with the same perforation pattern as the false bottom plate was inserted above the gravel before the fertilized eggs were placed into the incubators. To the top of this plate, a fiberglass insect mesh was attached with thermoplastic cement. This plate prevented embryos from falling into the gravel and also allowed easy access to embryos for sampling while minimizing mortality of unsampled embryos. Water was allowed to run through the gravel for 7 d before embryos were introduced.

In September 1995, eggs from 20 females returning to the Armin F. Koernig Hatchery in Prince William Sound were removed into separate one-gallon reclosable freezer bags, and 5 ml of milt from each of 20 males was placed into separate 15-ml capped centrifuge tubes. Gametes were

placed on wet ice and flown to the ADF&G Genetics Laboratory in Anchorage. Within 10 h of gamete collection, 20 single-pair matings were performed. For each mating, fertilized eggs were divided into two groups; half were placed in an incubator with oiled gravel, and half were placed in an incubator with unoiled gravel. The first 10 matings were placed into incubators without the top plate in place. These matings provided mortality and abnormality data. The top plate was then inserted and the second set of matings were placed into the incubators above this plate.

Incubation temperature averaged 5.6° C and ranged from 5.0 to 6.5° C. Flow was maintained between 170 and 230 ml/min and was adjusted three times a week. A prophylactic treatment to control fungus, consisting of 13% NaCl, was administered twice a week for 1 h. Dissolved oxygen concentrations in incubator effluents were measured during hatching and were all above 90% of saturation. Embryos from two families were sacrificed after an 85-day exposure.

<u>DNA extractions and PCR amplification</u>
DNA was isolated from a total of 40 embryos and their 4 parents. This represents the offspring of two families of pink salmon that were experimentally exposed under controlled laboratory conditions to gravels that were either treated with weathered Prudhoe Bay crude oil or were untreated. DNAs were isolated from whole embryos at ADF&G Genetics Laboratory or from embyros supplied to NYUMC using C-TAB buffer as described in Wirgin et al. (1990). DNA concentrations and purity were determined spectrophotometrically at 260 and 280 nm.

K-*ras* exon 1 was PCR amplified using the forward primer (H01) 5'-ATGACGGAATACAAGCTG-3' and the reverse primer (C37) 5'-CTCGATGGTGGGGTCATATT-3' described for rainbow trout *Oncorhynchus mykiss* (Fong et al. 1993). The PCR reaction mix contained 2.5 mL of 10 x reaction buffer (Promega), 1.5 mL of 25 mM $MgCl_2$, 200 mM of each dNTP, 1.0 mL of each primer (30 mM stock), 10-100 ng of template DNA, and ddH_20 to a final reaction volume of 25 mL. The reaction mix was denatured for 5 min at 94° C and 1.5-2.5 U of *Taq* I polymerase (Promega) was added. Amplification was for 40 cycles as follows; denaturation at 94° C for 1 min, annealing at 48° C for 1 min, and extension at 72° C for 1 min, followed by a final extension at 72° C for 7 min. The K-*ras* exon 2 sequence was amplified using the forward primer (H38), 5'-GACTCGTACAGGAAGCAGGT-3' and the reverse primer (C79) 5'-CATGGCGCTGTACTCCTCCT-3' (Fong et al. 1993). PCR reaction conditions were identical to those for exon 1.

<u>Sequence analysis of the K-*ras* gene</u>
PCR products were separated electrophoretically in 2.0% low melting point agarose gels (Nu-Sieve GTG, FMC Corp.), the 111 bp amplicon from exon 1 and 90 bp amplicon from exon 2 were excised in agarose plugs and sequenced directly (Kretz and O'Brien, 1993) using the Cyclist™ Taq Sequencing Kit (Stratagene) with ^{35}S dATP at an annealing temperature of 62° C. All samples were sequenced at least four times; twice with the forward and twice with the reverse primers described above. Reaction products were resolved in 6% polyacrylamide/7M denaturing urea gels. Gels were fixed, air-dried, and exposed to X-ray film at room temperature for 1-5 days.

3'-primer mismatch analyses

3'-mismatch primers were designed based on initial results from direct sequence analysis of K-*ras* exons 1 and 2 from pink salmon DNA. Mismatch primers were developed for codons 12 and 13 in exon 1 and codon 61 in exon 2 (Table 1). 3'-mismatch primers were designed to exactly correspond to the mutations of the pink salmon K-*ras* variant alleles previously identified by sequence analyses. As a result, two mismatch primers were designed each for codons 12 and 13, and one mismatch primer was designed for codon 61 (Table 1). In addition, wild-type pink salmon K-*ras* 3' primers were developed in order to maximize annealing temperature during PCR.

Initially, PCR conditions were optimized with the mutated and wild-type primers to empirically define the maximum annealing temperature during PCR that would permit amplification of salmon genomic DNA with sequence perfectly complementary to that of the primers, but would not support amplification with primers containing a single 3' mismatched nucleotide. It was anticipated that the wild-type primer would successfully amplify the wild-type allele, but not the mutated allele under these highly stringent annnealing conditions. Conversely, use of the mutated primer would successfully amplify the mutated allele, but not the wild-type allele. These expectations were confirmed empirically in experiments with pink salmon DNAs carrying exclusively the wild-type alleles and mosaics with both wild type and mutant alleles (determined by sequence analysis).

Once these conditions were defined, the primer mismatch assay was used to more sensitively determine the frequency of mutations at codons 12, 13, and 61 in genomic DNA isolated from salmon embryos. DNA from each embryo was amplified with a 5' primer that corresponded exactly to wild type K-*ras* sequence and a set of 3' primers that contained the wild type and mutated 3' K-*ras* sequences. Annealing temperatures for mismatch assays at codons 12, 13, and 61 were 63° C, 62° C, and 61° C, respectively. Each set of PCR reactions included a positive control (salmon DNA with a mutation), a negative control (salmon DNA with no mutation), and a water control (all the PCR reactants with the exception of template DNA). Each reaction was run three times.

Approximately, 200 ng of DNA was used as template in the 3'-primer mismatch assays. Reactions were in 50 µL volumes containing 5 µL of 10 x reaction buffer (Promega or Boheringer Mannheim), 1.5 mM $MgCl_2$ (final concentration), 1 µM of each primer (final concentration), and 2 U of *Taq* DNA polymerase (Promega or Boehringer Mannheim). Reactions were denatured at 95° C for 5 min and maintained at 80° C until the addition of dNTPs. 200 µM (final concentration) of dNTPs (Pharmacia) were added to start the reactions at 80° C and cycling was started immediately. The cycling parameters were 95° C for 1 min, 61° C for 1min, 72° C for 1 min for 40 cycles followed by a final extension at 72° C for 7 min. Fifteen µLs of reactions were electrophoresed in 1.8% agarose gels and DNA fragments were visualized with ethidium bromide staining and photographed.

Data analysis
The statistical significance of differences in the frequencies of K-*ras* alleles between oil and non-oil treated embryos were evaluated using X^2 contingency analysis in the Statview™ SE Package from Abacus Concepts, Inc. 1988.

RESULTS

We used K-*ras* oligonucleotide primers originally described for rainbow trout to successfully PCR amplify exons 1 and 2 of the pink salmon K-*ras* oncogene. PCR products of 111 bp and 90 bp were obtained from amplification of pink salmon K-*ras* exons 1 and 2, respectively, and these were characterized using manual direct DNA sequencing methods with ^{35}S radiolabel. The wild type pink salmon sequence for K-*ras* exon 1 differed by 2 and 14 nucleotide substitutions, respectively, from that reported for rainbow trout and Atlantic tomcod. For K-*ras* exon 2, pink salmon wild type sequence differed by 2 and 7 nucleotide substitutions, respectively, from that of rainbow trout (Accession #M73690) and Atlantic tomcod (Accession #M73690) (Figure 1).

In total, for K-*ras* exon 1, sequence data was obtained for 18 oiled embryos, 20 of their non-oiled siblings, and the parents of families 2A and 2B (Fig 2; Table 2). Mutations were observed at both codons 12 and 13; sites that are frequently mutated in chemically treated rodents and in aflatoxinB_1 and PAH-treated rainbow trout embryos (Bailey et al. 1996). At codon 12, a GGA to GAA transition was observed which results in a deduced glycine to glutamic acid substitution. At codon 13, a GGT to AGT transition was observed which results in a deduced substitution of serine for glycine residues. In total, 9/10 and 4/10 oiled offspring in family 2A exhibited mutations at codons 12 and 13, respectively. In total, 5/8 and 2/8 oiled offspring from family 2B exhibited mutations at codons 12 and 13, respectively. None of the non-oiled offspring from families 2A or 2B exhibited these mutations in exon 1, nor were they observed in the four parents of the two families. The frequencies of mutated K-ras alleles were significantly higher in oiled than non-oiled embryos both at codon 12 (X^2= 24.6, $p < 0.0001$) and codon 13 (X^2 = 7.9, $p < 0.005$).

In total, for K-*ras* exon 2, sequence data was obtained for 20 oiled embryos, 20 non-oiled siblings, and the 4 parents of the two families (2A and 2B). Six of the 20 oiled embryos (30%) exhibited evidence of mutations at nucleotide position 1 of codon 61. This mutation was a CAG to AAG transversion (Fig. 2) and resulted in a deduced glutamine to lysine substitution. This mutation is frequently observed in experimentally induced rodent liver tumors (Reynolds et al. 1987). None of the 20 non-oiled siblings from the two families exhibited this mutation nor was it present in the four parents of the two families. The frequency of this mutation was significantly higher in oiled than in non-oiled embryos (X^2= 7.06, $p < 0.01$).

To confirm the results from sequence analysis and perhaps to more sensitively screen for mutations at these sites, 3'-mismatch assays were developed, optimized, and employed to screen for the frequency of these mutations in K-*ras* exons 1 and 2. Use of this approach confirmed all of the mutations that were originally detected with the DNA sequencing approach at codons 12, 13, and 61 in both families 2A and 2B (Table 2). At codon 12, the frequency of mutations detected by sequencing and the 3' mismatch approach were almost in perfect agreement.

Seventy eight percent of oiled embryos (14/18) revealed mutations by direct sequencing and 75% (15/20) of oiled embryos by the 3' mismatch assay. The frequency of mutations at codon 13 were lower, but both techniques once again provided similar results. DNA from 33% of oiled embryos (6/18) exhibited mutations with direct sequencing and 40% (8/20) by the 3' primer mismatch assay. At codon 61, the frequency of mutations revealed by direct sequencing was slightly lower than observed with the primer mismatch assay; 30% (6/20) with direct sequencing and 45% (9/20) with the primer mismatch assay. In total, all 10 oiled offspring from family 2A and 60% (6/10) of offspring from family 2B exhibited at least one mutation in K-*ras* as revealed by primer mismatch analyses. Thirty percent (6/20) of oiled offspring revealed mutations at all three mutable codons. In contrast, all 20 non-oiled siblings from families 2A and 2B, as well as their four parents, proved negative at all three codons using both assays.

DISCUSSION

Our results indicate that molecular genetic damage can occur frequently in pink salmon embryos that are experimentally exposed to weathered Prudhoe Bay crude oil. To our knowledge, this is the first demonstration of point mutations caused in feral organisms by laboratory or environmental exposure to environmentally-released crude oil. Cytogenetic alterations (anaphase aberrations and micronuclei) have previously been reported in embryos of pelagic Atlantic mackerel *Scomber scombrus* that were environmentally exposed in the New York Bight to a myriad of xenobiotics, including PAHs (Longwell and Hughes 1980). Furthermore, Pacific herring *Clupea pallasi* embryos and larvae that were environmentally exposed to *Exxon Valdez* oil (Hose 1996) or experimentally exposed to dilutions of the water soluble fraction of Prudhoe Bay crude oil (Kocan et al. 1993) also exhibited cytogenetic damage (anaphase aberrations). These cytogenetic alterations were associated with reduced juvenile viability and surviorship, thus it highly unlikely that these macro genetic lesions were transmitted inter-generationally. In contrast, intensive use of flow cytometry failed to detect elevated macrolesions in DNA from pink salmon embryos that were experimentally exposed to Prudhoe Bay crude oil under identical conditions as performed in our experiments (Habicht et al. in prep). It can be envisioned that single nucleotide substitutions, such as those we report for K-*ras* would not be acutely lethal and therefore potentially transmitted between generations.

It is highly unlikely that the K-*ras* mutations that we observed resulted from germ line polymorphisms that naturally occur in pink salmon populations based on several lines of evidence; 1) non-oiled siblings did not exhibit K-*ras* mutations, 2) the parents of embryos exhibiting mutations did not display mutated K-*ras* alleles, 3) mutations were always at *ras* codons that are commonly mutated in preneoplastic lesions and tumors in rodents and fish by chemical treatments (Balmain et al. 1984; Reynolds et al. 1987; Bailey et al. 1996) and in humans (Anderson et al. 1992), 4) the spectra of mutations that we observed at codons 12, 13, and 61 in pink salmon embryos were often consistent with those induced by PAH treatment in rodents and fish (Fong et al. 1993; Bailey et al. 1996). However, the fact that Prudhoe Bay oil was able to induce K-*ras* mutations in pink salmon embryos under controlled laboratory conditions does not necessarily imply that similar mutations were induced in embryos from wild populations of pink salmon in tributaries of Prince William Sound following the *Exxon Valdez* oil spill. Variation in exposure conditions due to physical factors (temperature, salinity, currents, etc.), differing profiles of weathered crude oil products in natural and experimental environments, possible differing uptake, metabolism, or clearance kinetics of xenobiotics by

embryos in natural and experimental systems, and interpopulation variation in susceptibility to K-*ras* mutations must be considered when evaluating the effects of *Exxon Valdez* oil on developing embryos in natural populations.

Activation of *ras* is most frequently observed in benign (Balmain et al. 1984) or preneoplastic lesions (Horio et al. 1996), and in histologically defined or frank tumors (Anderson et al 1992; Reynolds et al. 1987). Additionally, activation of *ras* (H-*ras* and K-*ras*) has been observed in rat mammary tissue (Kumar et al. 1990) and in mouse skin (H-*ras*) (Nelson et al. 1992) following chemical treatments and before the appearance of benign or neoplastic lesions. We believe our results to be one of the first demonstrations of K-*ras* mutations in normal tissues that do not eventually exhibit preneoplasia or neoplasia. This strongly suggests that the chemical agent, in this case weathered crude oil, was able to directly induce mutations at this critical gene locus. It has been suggested that mutations in K- or H-*ras* genes in preneoplastic lesions or tumors may be secondary effects of overall genomic instability which accompanies the later stages of the transformation process and therefore mutations may not be a direct consequence of chemical exposure. Also, evidence of generation of presumably spontaneous H-*ras* mutations in the absence of carcinogen treatment has been observed in rat mammary tissue (Cha et al. 1994). Our data suggest that K-*ras* mutations in pink salmon embyros result directly from xenobiotic exposure in oil-exposed pink salmon embryos.

Why were we able to detect genetic alterations in normal tissues, whereas, almost all other reports of *ras* activation have been observed in transformed tissues? Exposure of pink salmon to oil occurred shortly after fertilization when the number of cells in the developing embryo was small and during a period of development when metabolic pathways which activates aromatic hydrocarbon compounds is active in salmonid embryos (Fong et al. 1993). Therefore, if a mutation occurred in cells during this early stage of development, all cells descending from that lineage, and therefore a high percentage of the cell population in the developing embryo would carry the mutation and permits its detection. Additionally, the sensitivity of our techniques in detecting mutations probably also contributed to the high frequency of mutations that were observed.

Is this relatively high mutation rate that we observed in K-*ras* reflective of similar events at other loci in oil-treated pink salmon embyros? DNA from these same pink salmon embyros was also screened for mutations at the cytochrome *B* (cyt *b*) gene in mitochondrial DNA (mtDNA), selected exons of p53, and multiple microsatellite loci. These loci were selected for analysis because of their high mutational rates in other models exposed to environmental chemicals or radiation or in human tumors. For example, cyt *b* nucleotide sequence (Baker et al. 1996) and microsatellite diversity (Ellegran et al. 1997) was significantly higher in vole and barn swallow populations, respectively, in close proximity to Chernobyl than in more distant populations. Similarly, p53 is the most frequently mutated locus found in many human tumors (McMahon 1994). However, no evidence of an increased mutation rate was found at these loci in oil-exposed pink salmon embyros. These results indicate that sensitivity to damage differs among gene loci and that this sensitivity may be agent specific.

Is K-*ras* mutation in oiled pink salmon simply a marker of DNA damage or is it itself indicative of a threat to the health of salmon populations? Probably not, however it should be remembered

that all mutations observed in pink salmon embryos resulted in deduced amino acid substitutions, altered *ras* protein at sites critical to functional activity, and therefore of potential phenotypic importance. *Ras* mutations are most frequently associated with neoplasia and are thought to represent a critical early step in the initiation of some cancers. However, it is believed that *ras* activation is only one of many genetic alterations that must occur for the induction of many kinds of neoplasms (Fearon and Vogelstein 1990). Neoplasms are absent or infrequent in species of salmonid fishes (including pink salmon) with the exception of domesticated strains of rainbow trout which are highly susceptible to aflatoxin B_1 and PAH-induced tumorigenesis (Bailey et al. 1996) and feral lake whitefish *Coregonus clupeaformis* from the St. Lawrence River (Mikaelian et al. 1998). However, it should be remembered that the K-*ras* protein is critical in processing external messages to the cell, transmitting cellular signals from the cell surface to the nucleus, "cross talk" with multiple signal transduction pathways, and ultimately regulation of cell division and differentiation (McCormick 1993). Alterations in *ras* structure in a variety of taxa from baker's yeast to *Drosophila* have been shown to impact a variety of organismic responses including control of mitotic growth cycles (Egan and Weinberg 1993). Therefore, it is possible that alterations have phenotypic consequences in the development of pink salmon embryos.

Results from this study point to the possible utility of K-*ras* mutation as a screen for genetic damage in young life stages of wild fish that are exposed to environmentally released oil. However, several questions need first to be addressed. These include: 1) Is K-*ras* mutation in embryos from sensitive species dose-responsive to environmental oil exposures? 2) What is the extent of inter-specific variability in susceptiblity to K-*ras* mutation among wild fish species? Studies in other models have demonstrated inter-specific, inter-strain, and inter-tissue variation in susceptibility in activated *ras* in neoplasms induced by chemical agents (Anderson et al. 1992) and what is the functional importance of this genetic damage; 3) Did environmentally released *Exxon Valdez* oil actually incur mutations in K-*ras* in Prince William Sound pink salmon populations? 4) What is the potential of K-*ras* mutations induced in embryos to be transmitted intergenerationally?

Acknowledgements
This project was funded by the Exxon Valdez Trustee Council through project 9X191 and 9X196 and by the Alaska Department of Fish and Game. We also acknowledge NIEHS Center Grant ES00260 for support of this project. We thank J. McMahon for his attention to detail during aquaculture and J. Rosko and L. Maceda for their technical assistance.

REFERENCES

Anderson, M.W., S.H. Reynolds, M. You, and R.M. Maronpot. 1992. Role of proto-oncogene activation in carcinogenesis. *Environ. Health Perspect.* **98**:13-24.

Anderson, S., W. Sadinski, L. Shugart, P. Brussard, M. Depledge, T. Ford, J. Hose, J. Stegeman, W. Suk, I. Wirgin and **G. Wogan.** 1994. *Environ. Health Perspect.* **102**(Supple. 12):3-8.

Aspinwall, N. 1974. Genetic analysis of North American populations of the pink salmon, *Onchorhynchus gorbuscha*, possible evidence for the neutral mutation-random drift hypothesis. *Evolution* **28**:295-305.

Bailey, G.S., D.E. Williams and **J.D. Hendricks.** 1996. Fish models for environmental carcinogenesis:the rainbow trout. *Environ. Health Perspect.* **104** (Supple. 1):5-21.

Baker, R.J., R.A. Van Den Bussche, A.J. Wright, L.E. Wiggins, M.J. Hamilton, E.P. Reat, M.H. Smith, M.D. Lomakin and **R.K. Chesser**. 1996. High levels of genetic change in rodents of Chernobyl. *Nature* **380**:707-708.

Balmain, A., M. Ramsden, G.T. Bowden and **J. Smith**. 1984. Activation of the mouse cellular Harvey-*ras* gene in chemically induced benign skin papillomas. *Nature* **307**:658-660.

Baumann, P.C. and **J.C. Harshbarger**. 1995. Decline in liver neoplasms in wild brown bullhead catfish after coking plant closes and environmental PAHs plummet. *Environ. Health Perspect.* **103**:168-170.

Bickham, J. and **M Smolen**. 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. *Environ. Health Perspect.* **102** (Supple. 12):25-28.

Bue, B.G., S. Sarr, S.D. Moffitt and **A.K. Craig**. 1996. Effects of the *Exxon Valdez* oil spill on pink embryos and preemergent fry. In:*Proceedings of the Exxon Valdez Oil Spill Symposium*. Rice, S.D., R.B. Spies, D.A. Wolfe, and B.A Wright, editors. *American Fisheries Society Symposium* **18**:619-627.

Bue, B.G., S. Sharr and **J.E. Seeb**. 1998. Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the *Exxon Valdez* oil spill. *Trans. Am. Fish. Soc.*. **127**:35-43.

Cha, R.S., W.G. Thilly and **H. Zarbel**. 1994. *N*-nitroso-*N*-methylurea-induced rat mammary tumors arise from cells with preexisting oncogenic *Hras1* gene mutations. *Proc. Natl. Acad. Sci. USA* **91**:3749-3753.

Collier, T.K., M.M. Krahn, C.A. Krone, L.L. Johnson, M.S. Myers, S.-L. Chan and **U. Varanasi**. 1996. Oil exposure and effects in subtidal fish following the *Exxon Valdez* oil spill. *Proceedings of the Exxon Valdez Oil Spill Symposium*. Rice, S.D., R.B. Spies, D.A. Wolfe, and B.A. Wright, editors. American Fisheries Society Symposium **18**:671-683.

Egan, S.E. and **R.A. Weinberg.** 1993. The pathway to signal achievement. *Nature* **365**:781-783.

Ellegran, H., G. Lindgren, C.R. Primmer and **A.P. Moller**. 1997. Fitness loss and germline mutations in barn swallows breeding in Chernobyl. *Nature* **389**:593-596.

Fearon, E.R. and **B. Vogelstein**. 1990. A genetic model for colorectal tumorigenesis. *Cell* **61**:759-767.

Fong, A.T., R.H. Dashwood, R. Cheng, C. Mathews, B. Ford, J.D. Hendricks and **G.S. Bailey**. 1993. Carcinogenicity, metabolism and Ki-*ras* proto-oncogene activation by 7,12-dimethylbenz[a]anthracene in rainbow trout. *Carcinogenesis* **14**:629-635.

Guo, Z., Q. Liu and **L.M Smith**. 1997. Enhanced discrimination of single nucleotide polymorphisms by artificial mismatch hybridization. *Nature Biotechnol.* **15**:331-335.

Habicht, C., G.D. Miller, D. Evans, R. Heintz and **J.E. Seeb**. Intensive flow cytometry analyses do not detect macrolesions in pink salmon embryos following crude oil exposure. (Manuscript in prep.).

Hasegawa, Y., S. Takeda, S. Ichii, K. Koizumi, M. Maruyama, A. Fujii, H. Ohta, T. Nakajima, M. Okuda, S. Baba and **Y. Nakamura**. 1995. Detection of K-*ras* mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification. *Oncogene* **10**:1441-1445.

Hawkins, W.E., W.W. Walker and **R.M. Overstreet**. 1995. Carcinogenicity tests using aquarium fish. pp. 421-446 in G.M Rand (ed.). *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment.* Taylor and Francis, Washington, D.C.

Horio, Y., A. Chen, P. Rice, J.A. Roth, A.M. Malkinson and **D.S. Schrump**. 1996. Ki-*ras* and *p53* mutations are early and late events, respectively, in urethane-induced pulmonary carcinogenesis in A/J mice. *Mol. Carcinogenesis* **17**:217-223.

Hose, J.E. 1994. Large-scale genotoxicity assessments in the marine environment. *Environ. Health Perspect.* **102**(Supple. 12):29-32.

Kocan, R.M., J.E. Hose and **E. Biggs**. 1993. Herring embryo stage sensitivity to water soluble fraction of Prudhoe Bay crude oil. *Exxon Valdez Oil Spill* Symposium, pp. 250-253, Anchorage, AK.

Krahn, M.M., D.G. Burrows, W.D. MacLeod Jr. and **D.C. Malins**. 1987. Determination of individual metabolites of aromatic compounds in hydrolized bile of English sole (*Parophrys vetulus)* from polluted sites in Puget Sound, Washington. *Arch. Environ. Contam. Toxicol.* **16**:511-522.

Kretz, K.A. and **J.S. O'Brien**. 1993. Direct sequencing of polymerase chain reaction products from low melting temperature agarose. *Methods Enzymol.* **218**:72-79.

Kuchino, Y., F. Mori, H. Kasai, H. Inoue, S. Iwai, K. Miura, E. Ohtsuka and **S. Nishimura**. 1987. Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. *Nature* **327**:77-769.

Kumar, R., S. Sukumar and **M. Barbacid**. 1990. Activation of *ras* oncogenes preceding the onset of neoplasia. *Science* **248**:1101-1104.

Linden, O. 1978. Biological effects of oil on early development of the Baltic herring Clupea harengus membras. *Mar. Biol.* **45**:273-283.

Longwell, A.C. and **J.B. Hughes**. 1980. Cytologic, cytogenetic, and developmental state of Atlantic mackerel eggs from sea surface waters of the New York Bight, and prospects for biological effects monitoring with ichthyoplankton. *Rapp. P-v Reun Cons. Int. Expl. Mer.* **179**:275-291.

Maccubin, A.E. 1994. DNA adduct analysis in fish: laboratory and field studies. pp. 267-294. in: D.C. Malins and G.K. Ostrander (eds.) *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Lewis Publ., Boca Raton.

Malins, D.C., G.K. Ostrander, R. Haimanot and **P. Williams**. 1990. A novel DNA lesion in neoplastic livers of feral fish: 2,6-diamino-4-hydroxy-5-formamidopyrimidine. *Carcinogenesis* **11**:1045-1047.

Malins, D.C., N.L. Polissar, M.M. Garner and **S.J. Gunselman**. 1996. Mutagenic DNA base modifications are correlated with lesions in nonneoplastic hepatic tissue of the English sole carcinogenesis model. *Cancer Res.* **56**:5563-5565.

Marty, G.D., R.A. Heintz and **D.E. Hinton**. 1997. Histology and teratology of pink salmon larvae near the time of emergence from gravel substrate in the laboratory. *Can. J. Zool.* **75**:978-988.

McCormick, F. 1993. How receptors turn *Ras* on. *Nature* **363**:15-16.

McMahon, G. 1994. The genetics of human cancer: implications for ecotoxicology. *Environ. Health Perspect.* **102**(Supple. 12):75-80.

McMahon, G., L.J. Huber, M.J. Moore, J.J. Stegeman and **G.N. Wogan**. 1990. Mutations in c-ki-*ras* oncogenes in diseased livers of winter flounder from Boston Harbor. *Proc. Natl. Acad. Sci. USA*. **87**:841-845.

Mikaelian, I., Y. de Lafontaine, C. Menard, P. Tellier, J.C. Harshbarger and **D. Martineau**. 1998. Neoplastic and nonneoplastic hepatic changes in lake whitefish (*Coregonus clupeaformis*) from the St. Lawrence River, Quebec, Canada. *Environ. Health Perspect.* **106**:179-183.

Nelson, M.A., B.W. Futscher, T. Kinsela, J. Wymer and **G.T. Bowden**. 1992. Detection of mutant Ha-*ras* genes in chemically initiated mouse skin epidermis before the development of benign tumors. *Proc. Natl. Acad. Sci. USA* **89**:6398-6402.

O'Clair, C.E., J.W. Short and **S.D. Rice**. 1996. Contamination of intertidal and subtidal sediments by oil from the Exxon Valdez in Prince William Sound. In: *Proceedings of the Exxon Valdez Oil Spill Symposium*. American Fisheries Society Symposium **18**:61-93.

Olinski, R., T.H. Zastawny, M. Foksinski, A. Barecki and **M. Dizdaroglu**. 1995. DNA base modifications and antioxidant enzyme activities in human benign prostatic hyperplasia. *Free Radic. Biol. Med.* **18**:807-813.

Park, J-Y. K., M.K. Shigenaga and **B.N. Ames**. 1996. Induction of cytochrome P4501A1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or indolo(3,2-b)carbazole is associated with oxidative DNA damage. *Proc. Natl. Acad. Sci. USA* **93**:2322-2327.

Rand, G.M., P.G. Wells and **L.S. McCarty**. 1995. Introduction to aquatic toxicology. pp 3-67 in: G.M. Rand (ed). *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*. Taylor and Francis, Washington, D.C.

Reynolds, S.H., S.J. Stowers, R.M. Patterson, R.H. Maranpot, S.A. Aaronson and **M.W. Anderson**. 1987. Activated oncogenes in B6C3F1 mouse liver tumors: implications for risk assessment. *Science* **237**:1309-1316.

Rhein, T., M.L. DePamphilis and **H. Zorbas**. 1998. Identifying 5-methylcytosine and related modifications in DNA genomes. *Nucleic Acids Res.* **26**:2255-2264.

Stein, J.E., W.L. Reichert and **U. Varanasi**. 1994. Molecular epizootiology: assessment of exposure to genotoxic compounds in teleosts. *Environ. Health Perspect.* **102**(Supple. 12):19-23.

Van Beneden, R.J. and **G.K. Ostrander**. 1994. Expression of oncogenes and tumor supressor genes in teleost fishes. pp. 295-325 in: D.C. Malins and G.K. Ostrander (eds). *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*, Lewis Publ., Boca Raton.

White, R.D. and **J.J. Stegeman**. 1996. Inactivation of cytochrome P4501A1 by 3,3',4,4'-tetrachlorobiphenyl and the formation of reactive oxygen species. Abstract SETAC, Washington, D.C.

Wiedmer, M., M.J. Fink, J.J. Stegeman, R. Smolowitz, G.D. Marty and **D.E. Hinton**. 1996. Cytochrome P-450 induction and histopathology in preemergent pink salmon from oiled spawning sites in Prince William Sound. *Proceedings of the Exxon Valdez Oil Spill Symposium*. Rice, S.D., R.B. Spies, D.A. Wolfe, and B.A. Wright, editors. American Fisheries Society Symposium**18**:509-517.

Wirgin, I., D. Currie and **S.J. Garte**. 1989. Activation of the K-*ras* oncogene in liver tumors of Hudson River tomcod. *Carcinogenesis* **10**:2311-2315.

Wirgin, I., M. D'Amore, C. Grunwald, A. Goldman and **S.J. Garte**. 1990. Genetic diversity at an oncogene locus and in mitochondrial DNA between populations of cancer-prone Atlantic tomcod. *Biochem. Genetics* **28**:459-475.

```
                    1     H01                                                              70
Pink Salmon (wt)    ATGACGGAAT ACAAGCTGGT GGTGGTGGGG GCAGGAGGTG TGGGCAAGAG CGCGCTCACC ATCCAGCTCA
Pink Salmon (mt)    .......... .......... .......... ....*.*... .......... .......... ..........
Rainbow Trout       .......... .T........ .......... .......... .......... .......... ..........
AtlanticTomcod      .....A..G. .T........ .......... ..T.....C. .A........ ...AT.G... ..........

                    71                               C37         ↓          H38            140
Pink Salmon (wt)    TTCAGAACCA CTTTGTGGAT GAATATGACC CCACCATCGA GGACTCGTAC AGGAAGCAGG TGGTGATTGA
Pink Salmon (mt)    .......... .......... .......... .......... .......... .......... ..........
Rainbow Trout       ........A. .......... .......... .......... .......... .......... ..........
Atlantic Tomcod     .C........ T........C ..G....... .A........ .......... ..A....... .......A..

                    141                                                C79        201
Pink Salmon (wt)    TGGGGAGACG TGTCTGCTGG ACATCCTGGA CACAGCAGGT CAGGAGGAGT ACAGCGCCATG
Pink Salmon (mt)    .......... .......... .......... .......... ..*....... ...........
Rainbow Trout       C......... .......... .......... ...T...... .......... ...........
Atlantic Tomcod     C......... .....A.... .......... ......G..C .....A.... ...........
```

Fig. 1

Nucleotide sequence comparison of K-*ras* exons 1 and 2 among pink salmon, rainbow trout, and Atlantic tomcod. Mutated nucleotides in pink salmon embryo DNA are indicated by asterisks. The boundary between exons 1 and 2 is indicated by the arrow. Oligonucleotides primers H01, C37, H38, and C79 (Fong et al. 1993) used for PCR amplification and DNA sequencing are underlined.

DRAFT

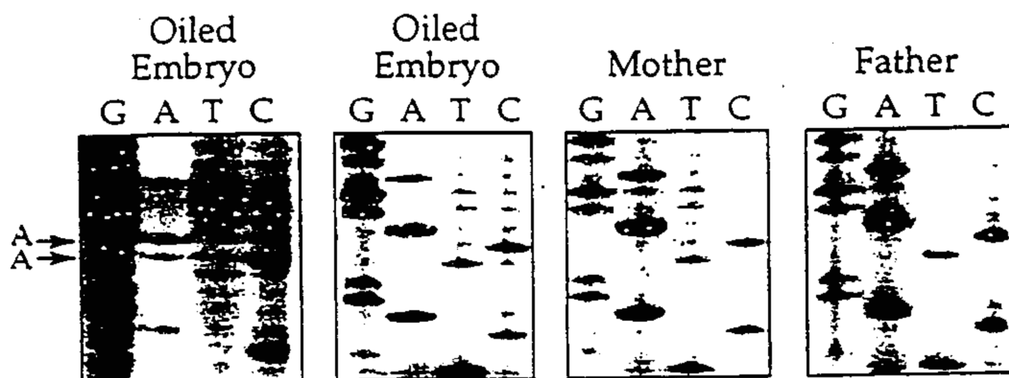


Fig. 2
An autoradiograph of a polyacrylamide gel exhibiting DNA sequence at codon 61 of the K-*ras* oncogene in two oiled pink salmon embryos and their two parents. DNA from oiled embryo in the left hand panel exhibits a C to A transversion, while the second oiled embryo and the two parents exhibit only the wild type sequence.

Table 1

DNA Sequences of Mismatch Wild Type (wt) and Mismatch Mutant (mt) PCR Primers Used to Amplify K-*ras* in Pink Salmon Embryos in 3' Mutant Mismatch Assays

Exon I	**Allele**			**DNA Sequence**
Upstream	Normal			5'-ATGACGGAATACAAGCTG-3' (H01)[1]
		Codon 12	Mismatch wt	5'-TGGTGGTGGGGGCAGG-3'
			Mismatch mt	5'-TGGTGGTGGGGGCAGA-3'
		Codon 13	Mismatch wt	5'-GTGGTGGGGGCAGGAG-3'
			Mismatch mt	5'-GGTGGTGGGGGCAGGAA-3'
Downstream	Normal			5'-CTCGATGGTGGGGTCATATT-3'(C37)[1]
Exon II				
Upstream	Normal			5'-GACTCGTACAGGAAGCAGGT-3'(H38)[1]
Downstream	Normal			5'-CATGGCGCTGTACTCCTCCT-3' (C79)
Codon 61	Mismatch wt	5'-GCGCTGTACTCCTCCTG-3'		
			Mismatch mt	5'-GGCGCTGTACTCCTCCTT-3'

[1] from Fong et al. (1993)

Table 2
Frequencies of Mutations at K-*ras* Exons 1 and 2 in Embryos from Two Families of Experimentally Treated Pink Salmon Based on DNA Sequencing and 3' Mutant Mismatch Results

Treatment	Family	Codon 12 Normal	Codon 12 Variant	Codon 13 Normal	Codon 13 Variant	Codon 61 Normal	Codon 61 Variant
DNA Sequencing Results							
Control	(2A)	10	0	10	0	10	0
Oiled	(2A)	1	9	6	4	6	4
Control	(2B)	10	0	10	0	10	0
Oiled	(2B)	3	5	6	2	8	2
3' Mutant Mismatch Results							
Control	(2A)	10	0	10	0	10	0
Oiled	(2A)	1	9	5	5	4	6
Control	(2B)	10	0	10	0	10	0
Oiled	(2B)	4	6	7	3	7	3