

*Exxon Valdez* Oil Spill  
Restoration Project Annual Report

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

Restoration Project 95191A-2  
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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**Study History:** The molecular genetics component of Restoration Project 95191A, the study of persistent mortality of pink salmon embryos, was initiated in 1992 as Restoration Study Number 60C. Project 60C and the 9x191 series of Restoration 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 Restoration Projects 94191 and 95191. Final reports were completed for Fish/Shellfish Study Number 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). The annual report for 95191A-1 (field component) was submitted April 1996. This is the annual report for the laboratory component of 95191A which focuses on molecular genetic screens for genetic damage.

This project series supported preparation of all or part of the following manuscripts:

- Bue, B.G., S. Sharr, and J.E. Seeb. Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the *Exxon Valdez* oil spill. (Approved by ADF&G editorial review and submitted to Transactions of the American Fisheries Society, September, 1996).
- Habicht, C., S. Sharr and J.E. Seeb. Coded wire tag placement affects homing precision of pink salmon. (Revised for ADF&G editorial review, July 1996).
- 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. (Substantially revised manuscript, approved by ADF&G editorial review, and submitted to Archives of Environmental Contamination & Toxicology, September, 1996).
- 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.
- Olsen, J.B., J.K. Wenburg and P. Bentzen. 1996. Semi-automated multilocus genotyping of Pacific salmon (*Oncorhynchus spp.*) using microsatellites. *Molecular Marine Biology and Biotechnology* (Accepted by journal and in press for November issue).

**Abstract:** We used flow cytometry and haploid androgenesis to test the hypothesis that incubation of pink salmon embryos in an oiled substrate induces genetic damage. Further, we developed new and adapted existing methods to test for microlesions in the DNA of pink salmon. 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 overall in either cytochrome *b* or the region of tumor suppressor gene *p53* reported to be hypervariable in other species. We tested for amplification and expression in pink salmon at 35 microsatellite loci. A pilot study of four microsatellite loci showed no change of allelic expression among tissues between oiled and non-oiled treatments. Finally, we report development of a multi-locus DNA fingerprinting assay using *Tc1* and *Sma1* primers generated from other species that should prove useful for detecting small deletions or insertions in pink salmon DNA.

**Key Words:** Crude oil, cytochrome *b*, embryo mortality, *Exxon Valdez* oil spill, flow cytometry, genetic damage, mtDNA, mutation, *Oncorhynchus gorbuscha*, pink salmon, Prince William Sound, *p53*, *Tc1/Sma1*.

**Project Data:** The following data are available upon request of the custodian at the Alaska Department of Fish and Game, Genetics Laboratory (907) 267-2454.

<i>Type of data</i>	<i>Format of Data</i>	<i>Custodian</i>
Flow cytometry	Multicycle software - Phoenix Flow Systems.	Chris Habicht
DNA fragment analysis	Genotyper software (Macintosh formatted) - Applied Biosystems, Inc.	Eric Kretschmer Jeff Olsen Ben Greene
Mortality data of oiling experiment	Excel spreadsheet	Chris Habicht
Mortality data of haploid androgenesis	Excel spreadsheet	Gary Thorgaard
DNA sequencing analysis	Sequencing Analysis software (Macintosh formatted) - Applied Biosystems, Inc.	Eric Kretschmer
Manual sequencing	Excel spreadsheet	Ike Wirgin

**Citation:**

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## TABLE OF CONTENTS

	<u>Page</u>
LIST OF FIGURES .....	6
LIST OF TABLES .....	7
LIST OF APPENDICES .....	8
EXECUTIVE SUMMARY .....	9
INTRODUCTION .....	13
Flow Cytometry .....	14
Androgenesis .....	15
Development of Mutation Screens for Microlesions in Pink Salmon .....	15
Mitochondrial DNA .....	15
Tumor Suppressor Genes and Oncogenes .....	16
Microsatellites .....	17
DNA fingerprinting .....	17
OBJECTIVES .....	17
METHODS .....	17
Controlled Matings and Oilings .....	18
Flow Cytometry .....	19
Androgenesis .....	19
Polymerase-Chain-Reaction Based Screens for Microlesions .....	20
DNA sequencing of cytochrome <i>b</i> and <i>p53</i> .....	20
Microsatellites .....	22
Tcl/Smal fingerprinting .....	22
RESULTS .....	22
Survival and Abnormality .....	22
Flow Cytometry .....	22
Androgenesis .....	22
Screens for Microlesions .....	23
Cytochrome <i>b</i> .....	23
Tumor suppressor gene <i>p53</i> .....	23
Microsatellites .....	24
Tcl/Smal fingerprinting .....	24
DISCUSSION .....	24

Gene Detection Using the Polymerase Chain Reaction .....	26
LITERATURE CITED .....	29

## LIST OF FIGURES

	<u>Page</u>
1. Comparative nucleotide sequences of cytochrome <i>b</i> .....	46
2. Comparative nucleotide sequences for exons 7 - 10 of <i>p53</i> .....	47

## LIST OF TABLES

	<u>Page</u>
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 . . . . .	40
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 . . . . .	41
3. Parental haplotypes for nt 486 at the 5' end of codon 162 of cytochrome <i>b</i> sequenced with primer H15498. Number of progeny, incubated in oiled and control substrate, with A and G . . . . .	42
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 <i>p53</i> primers used . . . . .	43
5. Genotypes of fish, originating from the Little Port Walter (LPW) experiment, corresponding to base pair 1,054 of <i>p53</i> in rainbow trout . . . . .	44
6. Genotypes corresponding to base pair 1,054 of <i>p53</i> in rainbow trout cDNA . . . . .	45



**LIST OF APPENDICES**

	<u>Page</u>
A. Intensive Flow Cytometry Analyses do not Detect Macrolesions in Pink Salmon Embryos Following Exposure to Crude Oil . . . . .	48
B. Analysis of Deleterious Mutations in Pink Salmon using Haploid Androgenesis . . . . .	68
C. An Initial Evaluation of Molecular Genetic Damage to Oiled Pink Salmon Populations in Prince William Sound . . . . .	85
D. Use of Microsatellites in Two Genetic Studies of Alaskan Salmon ( <i>Oncorhynchus spp.</i> ) . . . . .	92
E. SINE and Transposon Sequences Generate High-Resolution DNA Fingerprints: Screening for Genetic Damage in Pink Salmon ( <i>Oncorhynchus gorbuscha</i> ) Exposed to Prudhoe Bay Crude Oil . . . . .	98

## EXECUTIVE SUMMARY

Restoration project 95191A 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 has been examined since the fall 1992. This report focuses upon work performed between October 1, 1994 and June 30, 1996.

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 naturally occurring 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 examined 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 smaller than 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 suggests 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.

Loss of microsatellite heterozygosity is reported to be a potentially useful indicator of genetic disease in some instances, so we initially screened nine microsatellite loci developed in other salmonid species for interspecific priming with pink salmon. We found that four of these, One $\mu$ 1, One $\mu$ 11,  $\mu$ Sat60,  $\mu$ Sat73 worked well. We then screened oiled and non-oiled populations from the Little Port Walter experiments at these four loci for alteration in allelic expression in heart, liver, kidney and spleen. No loss or gain of alleles was detected. Subsequent screen by one of us (JBO) provided an array of additional microsatellite markers for detection of genetic variation in pink salmon.

Because existing techniques for mutation detection are generally limited to screening specific, preselected genomic targets, this year 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 *Sma*I 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.

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. Results from matings that we conducted in 1993, with gametes from fish returning to oiled and reference streams, provide strong evidence that genetic damage affected pink salmon populations oiled in 1989, but documenting specific chromosome breaks or DNA mutations is proving to be elusive. The future of this project will include an intensive focus on the most promising strategies identified in FY 1995 and additional support for the Trustee Council-funded gene mapping studies during FY 1996 and 1997.

The promise of the different gene-detection strategies has evolved rapidly with the evolution of technologies and the increased understanding of genotoxic response. Single-base mutations continue to be implicated in a variety of germline disease, so completion of the cytochrome *b* and *p53* assays started on families oiled in project 95191A will remain a high priority.

Additionally, site-specific point mutations two exons within the oncogene *Ki-ras* were recently implicated as a genotoxic response of rainbow trout exposed to polycyclic aromatic hydrocarbons; therefore, we will further screen the same individuals for mutations at those nucleotides.

Loss of microsatellite heterozygosity, valuable in oncogene research, appears to be a cost intensive approach based upon our initial effort. That and a negative opinion from one peer reviewer led us to decide not to scale-up microsatellite assays for mutation screens in FY 1996. However, we will transfer the microsatellite technology adapted here to project series 9x196 and 9x255 where it shows substantial promise as a gene-detection technique valuable for population genetics studies; the same markers will be used in the project 9x190 series for gene mapping.

The *Tcl/SmaI* fingerprinting, given the advantages described above, has the major limitation that it is blind to point mutations. We will complete the screening of existing families for systematic variation (gain or loss of fragments), but the primary application of this approach will be to provide a large number of markers for the mapping of the pink salmon genome project 9x190. Additional markers for this purpose can also be developed using additional primers available in the SINE family.

## 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 have been shown to damage chromosomes (Longwell 1977; McBee and Bickham 1988; Hose et al. 1996). 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. *in review*), 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. *in review*). 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 Roberts et al. 1993; Wiedmer et al. 1996).

The focus of this component of Project 95191 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; Orner 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 automated-DNA-sequence based screens for mutations in pink salmon including sequencing of the mitochondrial DNA (mtDNA) locus cytochrome *b* and the nuclear DNA (nDNA) tumor suppressor gene *p53*, 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 I 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 in 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 *Ki-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 two hypotheses relating to the presence of genetic damage in pink salmon embryos exposed to Prudhoe Bay crude oil:

- 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.

Additionally, an important objective this year was to develop, in pink salmon, assays to determine if microlesions are more common in those 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 variables 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

This phase of the project was largely developmental in FY 1995. In some cases primers were available for PCR amplification of templates used in fragment polymorphism analyses of population structure, but these templates were too large for use in a cycle-sequencing mutation screen. 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 Genra 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<sub>2</sub>, 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<sub>2</sub>O, 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.

## Tcl/SmaI fingerprinting

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

## **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  $\alpha = 0.05$ .

## 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.

## Tcl/Smal fingerprinting

See Appendix E.

## **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 and 2) androgenetic progeny from males exposed to crude oil during development have faster mortality rates than androgenetic progeny from males not exposed. Additionally, an important goal this year was to develop, in pink salmon, assays to determine if microlesions are more common in those 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. Sharr et al. (1994a and 1994b) and Seeb et al. (1995) 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. Sharr et al. (1994a) 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; Hose et al. 1996); documentation of germline damage is yet to be reported.

An alternative to the genetic damage hypothesis is that observed differences in embryo mortality are due to environmental variation. 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.

However, results from the controlled incubation experiments conducted at AFK Hatchery indicate that the basis for the differences in embryo mortality observed in the field was not due to environmental variation in the incubation environment (Seeb et al. 1995). 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. We believe this to be unlikely; the controlled oiling experiment conducted by the National Marine Fisheries Service will provide laboratory evidence to further clarify interpretation of these field data.

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. 1996).

Our flow cytometry analysis of pink salmon embryos and larvae exposed to crude oil and its water soluble fraction 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 review in Bailey et al. 1996). 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, *One $\mu$ 1*, *One $\mu$ 11*,  *$\mu$ Sat60*, and  *$\mu$ Sat73*, 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, this year 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). Recent 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 Fromental 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 common solution and the approach that we will use is to use the sequencing assay to identify candidate sites and then design a more sensitive assay such as mismatch analysis (MMA) to re-examine treatment individuals for the presence of the mutations at that site (Bailey et al. 1996).

First, candidate sites need to be identified. The exons of *p53* are still important candidates because of the mutability and response to carcinogens observed other taxa (Williams et al. 1994; Mayr et al. 1995; Sagartz et al. 1996). We will expand our efforts to conduct a systematic screen of *p53* exons in FY 1996. Additionally, we will sequence the remaining 395nt region of the cytochrome *b* template, amplified by primers, LGL-765 and H15498, in the same individuals.

Finally, the *ras* family of oncogenes offer an exciting opportunity for mutation study in fish (Rotchell et al. 1995). The *ras* oncogenes (*H-ras*, *Ki-ras*, and *N-ras*) in humans and rodent models have been demonstrated to be sensitive to mutations induced by environmental PAHs. Mutations in these genes have been correlated with alterations in cellular signal transduction pathways and the prevalence of various tumors. Mutations almost invariably occur within exon 1 at codons 12 and 13 and within exon 2 at codons 59 and 61 through 63. Site-specific mutations within the *Ki-ras* oncogene at these same codons have also been reported in both feral and domesticated fish models which are exposed to genotoxic agents (Hendricks et al.

1994; Bailey et al. 1996). Consequently we believe, if *Exxon Valdez* crude oil is causing mutations in pink salmon, that a focus of our effort on sequence detection at these codons is appropriate.

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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	15.9	0.2	$P = 0.012$	$P = 0.016$

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		<i>P</i> 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 Fromental 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.

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

Rainbow Trout CTTCTAATAT CTCAGTCTGG TGAACCTTTG 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 ACTCATTCTG AACATCCATG CCAACGGAGC ATCTTTCTTT TTTATCTGTA
Pink Salmon G .....C.. .C.....T .....T..... .T..... .....T....
Pink Salmon A .....C.. .C.....T .....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 CTTGTAGGC TACGTCCTCC CGTGAGGACA AATATCATTG
Pink Salmon G .....T..... .....A..C..... .....G..C...
Pink Salmon A .....A..C..... .....G..C...

Rainbow Trout TGAGGGGCCA CTGTAATTAC AAACCTCCTC TCAGCTGTAC CATACGTAGG AGGCGCCCTA GTACAATGAA
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 TTTCACCTCC TATTCGCCCTT
Pink Salmon G .....C.. A.....T..... .C..... .....T.....T..
Pink Salmon A .....C.. A.....T..... .C..... .....T.....T..

Rainbow Trout CGTCATTGCA GCCGCTACGG TCCTTACCT 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 ATTAACCTCTG ATGCTGATAA AATCTCATTC CACCCTTACT TCTCATACAA AGATCTCCTA GGATTTCGTAG
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 TTTTTCACC AAATCTCCTA
Pink Salmon G .....T.. T..T..... ..A....C. .A..... ..CT..T..
Pink Salmon A .....T.. T..T..... ..A....C. .A..... ..CT..T..

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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 unoled 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)	ATTTGTCTTC	TCTTTTCTT	CATTTTACTA	ATTAATATAA	TAATAATATA	TGCCATTTAG	CAGACGCTTT
Pink Salmon							
Rainbow Trout (cDNA)	TATCCAAAGC	GACTTACAGT	CATGTGTGCA	TACATTCTAC	GTATGGGTGG	TCCCGGGAAT	CGAACCCACT
Pink Salmon							
Rainbow Trout (cDNA)	ACCCTGGCGT	TACAAGCGCC	ATGCTCTACC	AACTGAGCTA	CAGAAGGACC	ACAATTAGGA	AAAGCATCAT
Pink Salmon							
Rainbow Trout (cDNA)	TTGACTGAAT	TAAATAGAA	TTGACCCCAA	CCCTGGTCTT	GGGTTGACCC	CCAACATAATG	TCTTGTGCATA
Pink Salmon							
Rainbow Trout (cDNA)	GTATGACAGT	GGTGGGAAGT	TCCTCTCCCC	GTTCTTGCGAG	GGCAGCTC	CTGGGTGCGC	GCTCCTTTGA
Pink Salmon					GG•G.....	.....	.....
Rainbow Trout (cDNA)	GGTGCCTGTG	TGTGCCTGTC	CTGGTCGAGA	CAGGAAGACA	GAGGAGATCA	ACCTGAAGAA	CGACGAGGAG
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)	CCCGATGGTC	TTGGGGTTGG	GATTAACATA	AAAATGCCAA	AAAGAAAGAT	GTCAAGATCT	TTGGACAATT
Pink Salmon							
Rainbow Trout (cDNA)	GCACTTTAAA	GTTGGATGAT	GNCIGATAAA	GGTTGACATT	GACCTATGAC	CCTGAGTGTG	AGCAGTTCCT
Pink Salmon							
Rainbow Trout (cDNA)	CATCGTGTGT	GTCATGTTTG	TGTTCTTCTA	GCTATGAAG	GAGGCCTCCC	TGCTGCCCC	TCAGCCTGGG
Pink Salmon				A.....	.....	.....	•G•••A••
Rainbow Trout (cDNA)	GCCAGTAAGA	AG--ACCAAG	TC---CTCC	CCTGCTGTGA	GTGACGATGA	GATCTACACT	CTTCAG
Pink Salmon	.....	••ATTCATC•	••ACTC•••	.....	.....	.....	.....GTAA
Rainbow Trout (cDNA)	CTGCTCAGAG	AACAGACCAA	TGGAAGTTTT	TCTCGTCCCC	TTCAGAGATG	CCTAGTCACT	AGTCTGCTGT
Pink Salmon							
Rainbow Trout (cDNA)	TGGCTGTTTG	TGTATACTGT	GTGTAAGTGG	GCTCATCTGC	CCATCCAG••	AT TCGAGGGAAG	GAAAAATATG
Pink Salmon						.....	.....
Rainbow Trout (cDNA)	AGATGCTGAA	GAAGTTCAAT	GACAGTCTTG	AACTGAGTGA	GTTGGTGCCT	GTTGCCGACG	C
Pink Salmon	.....	.....	.....	.....S••	.....	.....	•



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

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### **Abstract**

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 DNA macrolesions 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 North Slope crude oil failed to detect macrolesions in our experiments. We had enough statistical power to detect treatment differences smaller than any of those reported in similar studies in the literature. We concluded that North slope crude oil, at concentrations up to 246 ng of polynuclear aromatic hydrocarbons per 1 g of tissue, does not cause macrolesions in pink salmon embryos.

### **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. *in press*). Observations of elevated mortalities of pink salmon embryos in cohorts not directly exposed to crude oil led to the hypothesis that the mortality may be caused by germline genetic damage.

A controlled mating experiment designed to assess the role of the environment in these field findings supports the germline genetic damage hypothesis. 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 (Sharr et al. 1994).

The clastogenic effect of crude oil and other, more potent, genotoxins was demonstrated by cytogenetic techniques that evaluate the frequency of 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 macrolesions 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 macrolesions 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 (McBee and Bickham 1988; Lamb et al. 1991; Custer et al. 1994). In a comparison of assays designed to detect genotoxicology, 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 that can be displayed in histograms. Various measurements from these histograms are used to assess genetic damage. These include the coefficient of variation ( $CV = \text{standard deviation}/\text{mean}$ ) 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).

We used flow cytometry in two controlled experiments to test for the presence of DNA macrolesions in pink salmon embryos and larvae exposed to crude oil. Experiment 1, a cooperative project between the Alaska Department of Fish and Game (ADF&G) and the National Marine Fisheries Service (NMFS), began in the fall of 1992 and was repeated in the fall of 1993 at the Little Port Walter Laboratory (LPW), Baranof Island, Alaska. This experiment tested the effects of five levels of oiled incubation substrate on various physiological and metabolic processes in developing pink salmon and provided larvae of known oil exposure for flow cytometry analysis.

Experiment 2, conducted at ADF&G's Genetics Laboratory in the fall of 1995, was designed to detect small differences in macrolesion frequency between embryos incubated in the effluent, above oiled and unoiled substrate. We narrowed the focus this experiment by examining fewer tissues from embryos exposed to only the unoiled and highest oil treatment in the previous study. This focus allowed us to increase the power of the experiment by increasing the number of embryos examined in each treatment. The experimental design also controlled for effects associated with family, date of sampling and analysis (Fisher et al. 1994), and tissue mixtures (Tiersch and Wachtel 1993). Finally, we held embryos in substrate

effluent until analysis thereby increasing the probability of detecting macrolesions (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 macrolesion rates were detected between oiled and reference embryos in either Experiment 1 or Experiment 2 even though the power of the latter experiment was capable of detecting differences smaller than any of the detected differences published in the literature for the same kinds of studies. We conclude that oil exposure during incubation does not induce macrolesions in pink salmon. We propose to continue to assess heritable genetic damage in pink salmon resulting from oil exposure, focusing instead on mutational alterations of DNA using more sensitive approaches that are capable of detecting microlesion damage.

## Materials and Methods

### Oil-Exposure and Tissue Sampling for Experiment 1

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 (Figure 1). Incubators were constructed of 60-cm sections of 15-cm-diameter polyvinylchloride (PVC) pipe stood 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 aluminum was fixed inside the pipe, providing a false bottom that held gravel in the incubator 3.8 cm above the bottom. The aluminum plate was perforated with 1.2-mm holes, which both diffused the water flow through the column of gravel and prevented pink salmon larvae from falling to the bottom of the incubator. 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 of gravel with a maximum diameter of 5.0 cm. Gravel was rinsed to remove fines prior to loading into incubators. Oil was weathered to drive off lighter polycyclic aromatic hydrocarbons (PAH's) by heating to 70°C in open flasks and stirring constantly for 18.5 h resulting in a 19.2% reduction in mass. In the 1992 experiment, gravel in each incubator was treated with weathered oil resulting in analyzed levels of 0.000, 0.007, 0.055, 0.622, and 4.510 g oil/kg gravel. In the 1993 experiment, weathered gravel from the highest level in the 1992 experiment was reused. This oiled gravel had water run through it for 230 d before being stored outdoors in an uncovered polyethylene container with drain holes during the summer of 1993. The analyzed level for this treatment was 2.860 g oil/kg gravel at the start of the 1993 experiment .

In 1992, gametes from 100 females and 100 males were taken from intertidal spawning pink salmon from Lovers Cove Creek, Baranof Island, Alaska. Eggs were pooled and milt was pooled. Fertilization was accomplished by mixing eggs and milt with effluent from their respective incubator. Fertilized eggs were then divided into the following number of

replicate gravel incubators: three 0.000, three 0.007, two 0.055, four 0.622, and four 4.510 g oil/kg gravel.

In 1993, gametes from 130 females and 130 males were taken from pink salmon from Lovers Cove Creek. Eggs were pooled and then divided into 130 aliquots. Each aliquot was fertilized by a single male. Fertilized eggs were pooled and subsequently divided into eight replicate incubators each for the 0.000 and the 2.860 g oil/kg levels.

Water supply to the incubators alternated between fresh and estuarine water to simulate the intertidal incubating environment of PWS. Incubators received fresh water from a nearby stream for 8 h followed by estuarine water (maximum salinity was 28‰) for 4 h. The water supplied a 1800-l head tank so that the salinity of water supplied to the incubators gradually changed over a 20-min period. Temperature was monitored daily and varied with the source; estuarine water ranged from 2.9 to 8.5°C, and fresh water varied between 0.8 and 9.2 °C. Water flow through each incubator was established prior to seeding the incubators with fertilized eggs and was monitored every other day to ensure a rate of 125 ml/min before the eyed stage of development and 200 ml/min thereafter. Dissolved oxygen concentrations in incubator effluent were monitored once every 2 weeks and never fell below 90% of saturation.

Samples were collected from the LPW experiments on two occasions. In April, 1993, groups of approximately 50 larvae incubated in each of the five 1992 treatments were shipped to the Anchorage laboratory. In April 1994, approximately 50 larvae incubated in gravel from the two 1993 treatments were also shipped to Anchorage. Samples of live larvae from all incubators within each treatment were taken without known bias. Samples were treated identically both years.

Upon arrival in Anchorage, all groups of larvae were incubated in individual screened cups within a 7°C freshwater recycle system for up to 7 d until flow cytometry analysis. Cups were numbered to correspond with the original sample numbers from LPW. To prevent bias, the relationship between cup number and oil-exposure level was unknown during subsequent tissue preparation and flow cytometry analysis.

Tissue preparation and flow cytometry analysis were conducted over a period of several days. On each day, all the treatment groups were analyzed but the order was randomized. Individual larvae within a cup were randomly chosen for dissection of gill, blood, liver, kidney, muscle and spleen tissue samples. A total of 60 to 100 tissue samples were analyzed per day. Microcentrifuge tubes containing prepared nuclei were labeled with cup, individual number and tissue type. Labeled tubes were randomized prior to flow cytometry analysis. Hydrocarbon analyses of the gravel, effluent, and embryonic tissue followed the methods of Heintz et al. (in press).

Oil-Exposure and Tissue Sampling for Experiment 2

Incubators were identical in design to those used in Experiment 1 except that a polypropylene plate with 4-mm-diameter holes on 8-mm centers was used as the false bottom rather than an aluminum plate. River gravel with maximum diameter of 5.1 cm, was washed and allowed to dry prior to oiling. Gravel was oiled with 5.7-g oil/kg gravel using unmodified 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 laid out in one layer and exposed to sun for 4 d before being placed in the incubators. Contrasting to Experiment 1, 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, thereby allowing easy access to embryos for sampling without inducing 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 10 females returning to 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 10 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, 10 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. Only one family was placed in each incubator.

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 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.

#### Tissue Preparation and Flow Cytometry

Several tissue types were analyzed during the experiments. In both experiments, 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. Additionally, in Experiment 1, individual tissues (blood,

gill, liver, kidney, muscle, spleen) were dissected from euthanized larvae.

Immediately after dissection, individual embryo and tissue samples were minced with two scalpels in 0.5 ml of nuclear isolation medium (NIM) (0.154 M NaCl, 10 mM Tris, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 106 mM MgSO<sub>4</sub> 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 size) to stain nuclei. Samples were vortexed for 3 s (Experiment 2 only), allowed to incubate at 2-3°C for 15 min, vortexed again for 3 s (Experiment 2 only), 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 triturated 3 times using a 26-g syringe (Experiment 1 only), 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 (Experiment 2 only).

Samples were analyzed using a Partec PAS II flow cytometer with optical filters for DAPI excitation. In Experiment 1, 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). Multicycle uses Gaussian distributions to fit G1 and G2 curves and a polynomial fitted by least squares to model the S-phase curve. In Experiment 2, we used the same methods for fitting the G1, G2, and S-phase curves, but added a debris curve to account for sliced nuclei. 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 calf thymus (Experiment 1) and trout erythrocytes (Experiment 2) (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 90 for calf thymus and 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 for Experiment 1 and 3.3 - 4.5 for Experiment 2. 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**

### **Experiment 1**

Data were normalized using a natural log transformation. One-way analysis of variance (ANOVA) was used to test G1 CV and G1/G2 ratio differences between oil-exposure groups for each brood year. A multivariate analysis of variance (MANOVA) was conducted on the G1 CV from blood, gill, and liver samples from the 1992 brood year individuals.

Flow cytometry histograms from blood, gill, liver, and spleen samples from the 1992 brood year were visually scored as typical or atypical. Atypical histograms were identified by the presence of a shoulder on either side of the G1 peak or the S-phase. The null hypothesis, that oiling and the occurrence of atypical histograms were independent, was tested by a chi-square test of independence.

## Experiment 2

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 10 blocks (based on family and date of sampling and analysis) which were treated as random effects. An average of 8.7 and embryos were 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. 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

### Experiment 1

Hydrocarbon analysis results are reported in Heintz et al. (in press).

No significant differences were observed for the mean CV of the G1 peak or the mean G1/G2 ratio of cells between groups in any of the tissues analyzed for both the 1992 and 1993 experiments ( $P > 0.074$  for all tests; Tables 1 and 2). No data for blood G1/G2 ratio are presented because fish erythrocytes, although nucleated, represent mature cells and do not possess a G2 phase of the cell cycle. For 51 individuals in the 1992 experiment no significant difference was found between oil exposure groups after MANOVA analysis of the G1 CV from blood, gill, or liver ( $P = 0.843$ ).

The frequency of typical and atypical histograms did not significantly differ between oil-exposure groups in any of the tissue histograms visually scored in the 1992 experiment ( $P > 0.302$  for all tests; Table 3).

### Experiment 2

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 4). 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 differences detected in similar studies, we would have had at least a 99% chance of detecting them for the G1 CV and G1/G2 variables and a 90% chance for the percentage of cells in the S-phase variable (see Figure 2 for G1 CV power).

## 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), rodents (McBee and Bickham 1988; Bickham 1990; Biradar and Rayburn 1996), turtles (Bickham et al. 1988; Lamb et al. 1991), and fish (Kocan et al. 1985; Kocan and Powell 1985; Jenner et al. 1990; Fisher et al. 1994).

However, we did not detect macrolesion 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 (Sharr et al. 1994). Incubation methods used in this study were not identical to natural incubation conditions in oiled sites within PWS. For example, our flushing rates and oiling concentrations may have been different than those found in PWS after the oil spill, and in Experiment 2, the embryos and fry were separated from the oiled gravel by a screen. However, the hydrocarbon content of embryos and fry from the high oiling levels in this study were similar to those observed in embryos and fry collected from oiled sites in 1990 (unpublished data - Ron Heinz will provide a citation here). Additionally, hydrocarbon levels in embryos and fry incubated above screens in a separate experiment conducted at LPW were not different from the levels found in embryos incubated within the gravel of the same incubators (Heinz et al. *in press*). Therefore, we conclude that the experimental incubation methods resulted in similar levels of



hydrocarbon uptake as those occurring in field-exposed fish.

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. If the crude oil did affect clastogenesis in Experiment 1, then DNA repair could be invoked to explain the absence of detectable macrolesions. However, organisms from Experiment 2 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).

We believe that if genetic damage is present in pink salmon incubated over oiled substrate, then it must be more subtle than macrolesions. Genotoxins also cause point mutations in oncogenes (reviewed in Van Beneden and Ostrander 1994; Hendricks et al. 1994), and PAHs are known to cause point mutations in rainbow trout (Fong et al. 1993). It may be that such point mutations are the cause of the germline damage apparent in the Prince William Sound pink salmon populations and that DNA-sequence-based analyses are necessary to detect them.

We are continuing our efforts to identify the type of genetic damage that may have been responsible for the increased mortality observed in our field and laboratory studies. We are designing experiments to detect potential microlesions in oil-exposed pink salmon embryos. One experiment will use haploid androgenesis (Armstrong and Fletcher 1983; G. Thorgaard, Washington State University, Department of Zoology, Pullman, WA, personal communication) to test for deleterious recessive mutations occurring in sperm from males originating in oil-contaminated streams. Other experiments will use the polymerase chain reaction and sequence-based analyses (Orita et al. 1989, cf., Fong et al. 1993; Hendricks et al. 1994) to detect nucleotide substitutions or deletions. Both of these approaches are more sensitive than either flow cytometry or traditional cytogenetic techniques at detecting microlesion damage while still allowing adequate sample sizes for statistical testing.

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Table 1. Summary of flow cytometry results from embryos of the 1992 brood year of Experiment 1. Data are from analysis of individual tissues from pink salmon exposed to five oil exposure treatments. The coefficient of variation (CV) of the G1 peak and the G1/G2 ratio of cells are presented for each exposure group along with the corresponding standard deviation (SD). Significant differences between oil-exposure groups were tested by one-way ANOVA of natural log transformed data.

Tissue	Oil Exposure (g/kg gravel)	Sample size	G1 CV	SD	<i>P</i> value	G1/G2 ratio	SD	<i>P</i> value
Blood	0.000	13	3.88	0.50	0.924	--	--	--
	0.007	12	4.26	0.90		--	--	--
	0.055	10	4.24	0.94		--	--	--
	0.622	13	4.02	0.84		--	--	--
	4.510	12	3.98	0.88		--	--	--
Gill	0.000	10	4.62	2.67	0.145	32.71	21.81	0.687
	0.007	8	7.01	5.72		24.14	14.19	
	0.055	7	3.47	1.52		31.89	26.58	
	0.622	7	4.29	1.60		27.03	23.26	
	4.510	7	8.54	7.53		38.17	24.28	
Kidney	0.000	4	2.73	0.61	0.171	20.74	15.29	0.507
	0.007	3	3.90	2.38		15.57	4.04	
	0.055	4	2.98	0.90		34.64	5.95	
	0.622	3	4.37	1.69		30.68	31.80	
	4.510	5	2.28	0.22		51.37	37.69	
Liver	0.000	12	2.76	0.51	0.171	84.91	62.01	0.973
	0.007	12	2.35	0.42		76.22	45.52	
	0.055	12	2.77	0.63		67.18	38.48	
	0.622	13	2.45	0.40		75.09	55.12	
	4.510	10	2.71	0.29		68.24	67.02	
Muscle	0.000	11	3.65	0.49	0.649	22.17	7.10	0.299
	0.007	9	3.90	0.52		16.35	5.37	
	0.055	9	4.00	0.78		16.39	5.90	
	0.622	12	3.98	0.58		19.71	13.34	
	4.510	8	3.81	0.42		18.15	7.67	

Table 1. Continued.

Tissue	Oil Exposure (g/kg gravel)	Sample size	G1 CV	SD	<i>P</i> value	G1/G2 ratio	SD	<i>P</i> value
Spleen	0.000	12	2.98	1.04	0.961	52.05	32.06	0.334
	0.007	11	3.25	0.88		41.27	18.34	
	0.055	10	3.35	1.25		30.17	25.24	
	0.622	12	2.99	0.38		51.88	25.03	
	4.510	11	3.16	0.67		51.76	28.16	

Table 2. Summary of Experiment 1 flow cytometry results from embryos of the 1993 brood year. Data are from analysis of individual tissues of control and oil-exposed pink salmon. The coefficient of variation (CV) of the G1 peak and the G1/G2 ratio of cells are presented for each exposure group along with the corresponding standard deviation (SD). Significant differences between oil-exposure groups were determined by one-way ANOVA of natural log transformed data.

Tissue	Oil Exposure (g/kg gravel)	Sample size	G1 CV	SD	<i>P</i> value	G1/G2 ratio	SD	<i>P</i> value
Blood	0.000	32	3.98	0.45	0.968	--	--	--
	2.860	30	3.99	0.49		--	--	
Gill	0.000	44	5.05	1.11	0.482	17.27	13.87	0.768
	2.860	41	5.20	1.04		16.45	5.78	
Kidney	0.000	52	4.15	0.79	0.074	22.21	13.11	0.177
	2.860	42	4.45	0.89		24.28	12.67	
Liver	0.000	43	3.38	0.68	0.569	38.23	24.56	0.565
	2.860	38	3.51	0.94		40.01	21.14	

Table 3. Experiment 1 results of visual scoring of flow cytometry histograms from the 1992 brood year . Tissue histograms were scored as appearing typical or atypical. Atypical histograms contained an obvious subpeak and shoulder on the G1 peak. Significant differences were tested by a chi-square test of independence.

Tissue	Oil Exposure (g/kg gravel)	Typical	Atypical	<i>P</i> value
Blood	0.000	6	3	0.302
	0.007	2	5	
	0.055	2	6	
	0.622	7	2	
	4.510	4	5	
Gill	0.000	6	3	0.516
	0.007	5	3	
	0.055	4	3	
	0.622	6	2	
	4.510	4	5	
Liver	0.000	6	0	0.306
	0.007	6	0	
	0.055	6	0	
	0.622	6	0	
	4.510	5	1	
Spleen	0.000	4	0	0.305
	0.007	3	0	
	0.055	3	1	
	0.622	3	1	
	4.510	1	1	



Table 4. For experiment 2, the 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)	<i>P</i> 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 5. The smallest significant differences in G1 CV (treated versus control) found in other studies using flow cytometry in relation to the power of Experiment 2.

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	No change detected. Had 90% chance to detect 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, 1.07
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	Triethylenemelamine	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, PCB's, heavy metals	Not reported	Night-heron	3.44	0.32
Biradar and Rayburn 1996	Herbicide (atrazine)	0.23 uM	Hamster ovary cell culture	3.89 for one chromosome	0.24 for one chromosome

Figure 1. Design of incubator used to expose pink salmon eggs to different levels of crude oil.

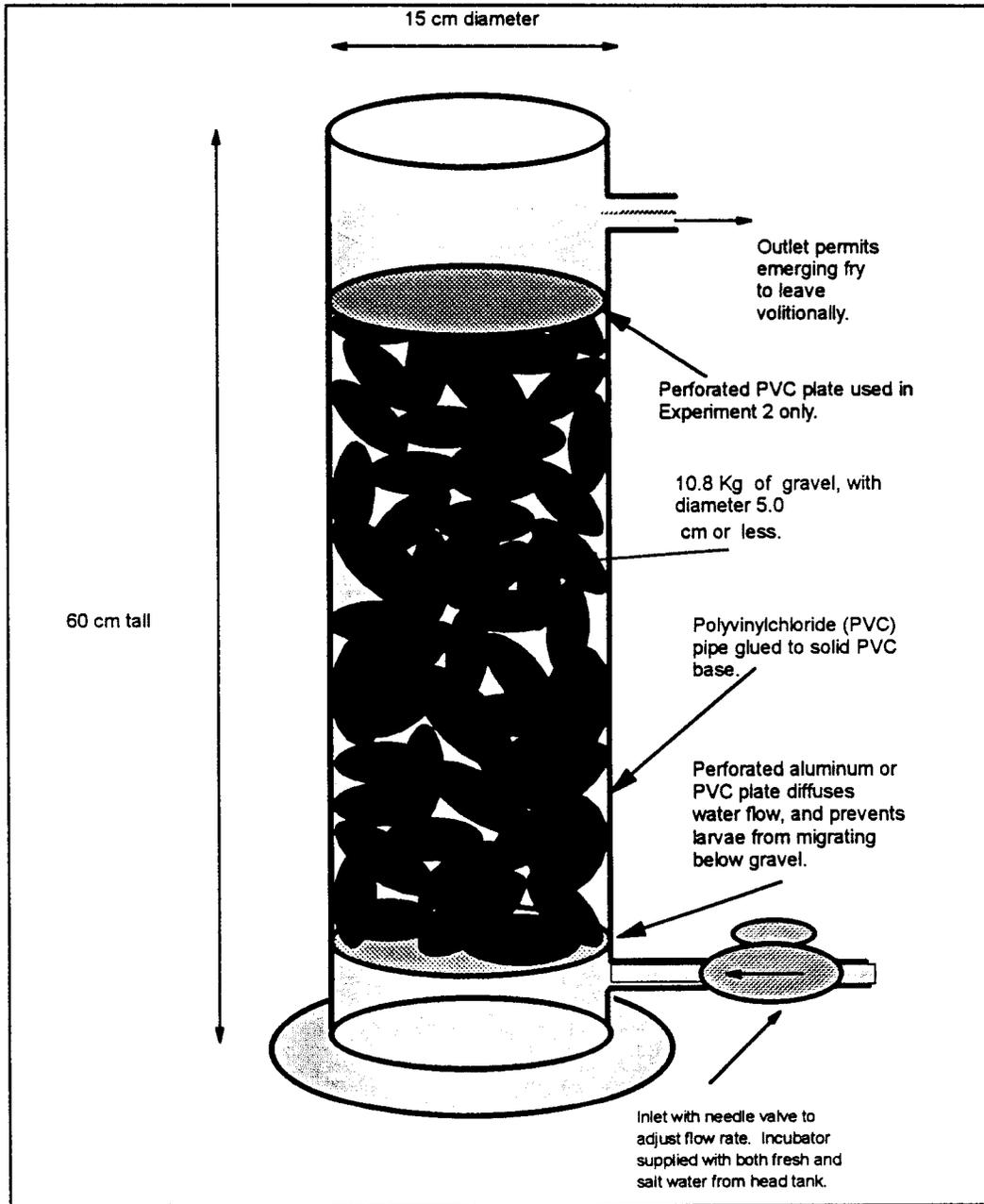
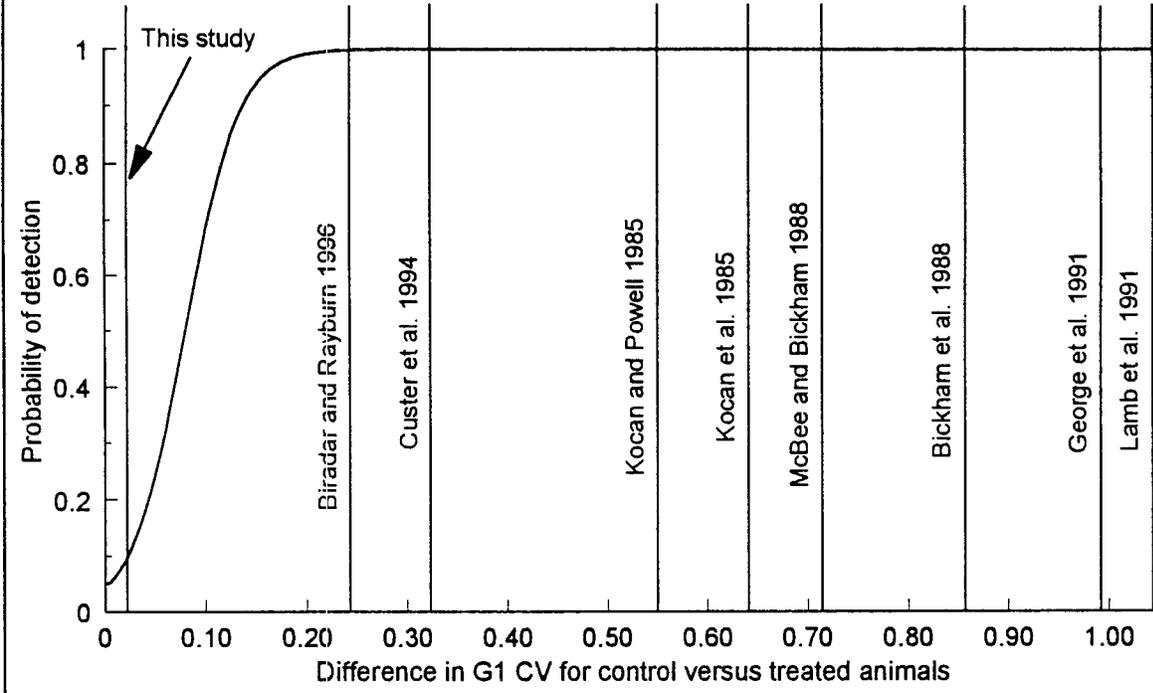


Figure 2. Power curve demonstrating the probability of detecting differences in CV of G1 peaks between oiled and control embryos at  $\alpha = 0.05$ . Superimposed are the smallest differences in the CV of the G1 peak for control versus treated animals detected by other authors using CV of the G1 peak to detect genetic damage. For more information on these authors' experiments see Table 5.



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

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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.

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.

<u>Survival Rate</u>			
<u>Treatment</u> (# males)	<u>Cleavage</u> <u>mean (+/-)</u>	<u>Streak</u> <u>mean (+/-)</u>	<u>Eyed</u> <u>mean (+/-)</u>
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 (0.007)
High oil (16 males)	0.495 (0.031)	0.216 (0.010)	0.060 (0.003)

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.

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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
o	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
o	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

## Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
F	High	25	82	0.30	A	1	Cleavage
G	Low	18	62	0.29	A	1	Cleavage
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
O	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

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
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
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
O	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
O	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

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
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
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
O	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

## Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
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
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

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
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
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

Appendix I

ID	Treatment	Survivors	Total	Percent	Rep	Experiment	Stage
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
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

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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

----- 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	0.23418	0.57178	0.90939	***
Low	- Control	0.32074	0.65308	0.98541	***
High	- Low	-0.90939	-0.57178	-0.23418	***
High	- Control	-0.11588	0.08129	0.27846	
Control	- Low	-0.98541	-0.65308	-0.32074	***
Control	- High	-0.27846	-0.08129	0.11588	

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.

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### 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-Marooof 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' - ctcccgtgaggacaaatgac - 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<sub>2</sub>, 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 <sup>35</sup>S using the Cyclist™ 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.



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Figure 1. Comparison of mtDNA cytochrome b sequences from #1 - control embryo, #1 - oiled embryo and OGB08. Alignment begins at position 175 of OGB08. Position of the polymorphic nucleotide site is highlighted in boldface.

	175
<u>1-Control</u>	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc
<u>1-Oiled</u>	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc
<u>OGB08</u>	tgtagcagct gcgatgacga aagggaatag aaagtgaaag gcgaaaaatc
	225
<u>1-Control</u>	gtgttagggt ggcgttatca acggagaatc cgcctcaaat ccattgtact
<u>1-Oiled</u>	gtgttagggt ggcgttatca acggagaatc cgcctcaaat ccaetgtact
<u>OGB08</u>	gtgttagggt ggcgftatca acggagaatc cgcctcaaat ccattgtact
	275
<u>1-Control</u>	agggcggcgc ccacataggg aacagcggaa agaaggtttgt
<u>1-Oiled</u>	agggcggcgc ccacataggg aacagcggaa agaaggtttgt
<u>OGB08</u>	agggcggcgc 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

<u>Incubator 2</u>	<u>Mutant C</u>	<u>Normal T</u>
1		+
2		+
3		+
4		+
5		+
6		+
7		+
8		+
9		+
10		+
11		+
12		+

B.

Oiled

<u>Incubator 2</u>	<u>Mutant C</u>	<u>Normal T</u>
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

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*(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 by projects 95191 and 95255. Study 2 actually preceded Study 1 and was conducted at Alaska Department of Fish and Game where Mr. Olsen identified four microsatellite loci that resolved well in pink salmon, and this study was funded 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 (*Oncorhynchus 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, One $\mu$ 1, One $\mu$ 11, One $\mu$ 14, Ots2, Ots6, Ssa4,  $\mu$ Sat60,  $\mu$ Sat73) 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. accepted and in press).

### *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/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 One $\mu$ 1, One $\mu$ 11,  $\mu$ Sat60  $\mu$ Sat73.

In phase two we compared heterozygosity at three loci (One $\mu$ 1, One $\mu$ 11,  $\mu$ Sat60) 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 embryogenesis.

### *Results and Discussion*

Results of phase one are summarized on table 3. Oiled and control individuals were monomorphic for One $\mu$ 1, One $\mu$ 11 and Sat73. The microsatellite  $\mu$ Sat60 was polymorphic, however the level heterozygosity among oiled and control individuals did not appear significantly different. Examples of electropherograms showing  $\mu$ Sat60 alleles from five individuals are shown in figure 1. In phase two, only  $\mu$ Sat60 was polymorphic in the four tissue types. One $\mu$ 1 and One $\mu$ 11 were monomorphic in all individuals regardless of tissue type. The level of heterozygosity at  $\mu$ Sat60 did not appear significantly different among tissues (Table 4). Examples of electropherograms showing the One $\mu$ 11 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**

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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<sup>a</sup>; (S) indicates the approximate size range (bases) of the PCR product<sup>b</sup>. 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
One $\mu$ 1	(58)-5	(58)-2-2	(58)-2-1	(58)-2-1	(58)-5	(58)-2-1,2
One $\mu$ 2	(58)-5	(58)-2-2,3	(58)-2-3,4	(57)-5	(57)-2-4	(58)-2-3,4
One $\mu$ 8	(58)-2-2	(55)-2-3	(58)-2-3	(55)-2-4	(55)-2-3	(55)-5
One $\mu$ 10	(57)-5	(57)-5	(57)-2-1,2	(57)-5	(57)-5	(57)-5
One $\mu$ 11	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2	(58)-1-2
One $\mu$ 14	(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
$\mu$ Sat15	(57)-5	(57)-5	(57)-5	(57)-5	(57)-5	(57)-5
$\mu$ Sat60	(60)-2-2	(60)-3-1	(60)-2-2	(57)-2-1	(57)-3-3	(60)-2-2
$\mu$ Sat73	(57)-2-2	(57)-2-2	(57)-3-2	(57)-2-2	(57)-2-1,2	(57)-5

<sup>a</sup> 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.

<sup>b</sup> 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

Table 3

## Heterozygosity at four microsatellite loci in oiled and unoiled pink salmon 1\

Locus	Treatment	No. Alleles	Genotype 2\							Total	H
			aa	ab	ac	ae	cc	cd	ce		
Str60	Control	3	27	0	15	4	2	0	0	48	39.6%
	Oiled-Dose6	5	23	1	22	0	0	1	1	48	52.1%
Str73 3\	Control	1	44	0	0	0	0	0	0	44	0.0%
	Oiled-Dose6	1	47	0	0	0	0	0	0	47	0.0%
One5D2 4\	Control	1	47	0	0	0	0	0	0	47	0.0%
	Oiled-Dose6	1	46	0	0	0	0	0	0	46	0.0%
One1A1	Control	1	48	0	0	0	0	0	0	48	0.0%
	Oiled-Dose6	1	48	0	0	0	0	0	0	48	0.0%

1\ DNA extracted from heart tissue.

2\ Allele sizes (bp) for each locus are as follows: Str60 a,b,c,d,e (110,112,114,116,118), Str73 a (136), One5D2 a (141), One1A1 a (103).

3\ Samples pc07,pc13,pc21,pc36,po37 did not amplify.

4\ Samples pc30,po02,po21 did not amplify.

Table 4

Heterozygosity at three microsatellite loci among four tissues from oiled pink salmon 1\

Locus	Tissue	No. Alleles	Genotype 1\							Total	H
			aa	ab	ac	ae	cc	cd	ce		
Str60	Heart	5	23	1	22	0	0	1	1	48	52.1%
2\	Kidney	5	23	1	22	0	0	1	1	48	52.1%
	Liver	5	22	1	21	0	0	1	0	45	51.1%
	Spleen	4	22	1	21	0	0	1	1	46	52.2%
One5D2	Heart	1	47	0	0	0	0	0	0	47	0.0%
3\	Kidney	1	47	0	0	0	0	0	0	47	0.0%
	Liver	1	48	0	0	0	0	0	0	48	0.0%
	Spleen	1	46	0	0	0	0	0	0	46	0.0%
One1A1	Heart	1	47	0	0	0	0	0	0	47	0.0%
4\	Kidney	1	48	0	0	0	0	0	0	48	0.0%
	Liver	1	47	0	0	0	0	0	0	47	0.0%
	Spleen	1	46	0	0	0	0	0	0	46	0.0%

1\ Allele sizes (bp) for each locus are as follows: Str60 a,b,c,d,e (110,112,114,116,118), One5D2 a (141), One1A1 a (103).

2\ Samples po10spleen,po13liver,po14liver,po28spleen,po29liver did not amplify.

3\ Samples po10spleen,po20kidney,po21heart,po28spleen did not amplify.

4\ Samples po10spleen,po19liver,po28spleen,po38heart did not amplify.

Figure 1. Electropherogram showing microsatellite locus *uSat60* (Str60) in five oiled pink salmon. Each peak represents a fluorescently labeled PCR product size fractionated using an Applied Biosystems Inc. ABI 377 DNA sequencer in GeneScan™ mode. The linear display depicts the emission intensity in relative fluorescent units (vertical axis) of each labeled fragment following excitation with an argon laser. Each allele is scored using ABI Genotyper™ ver 1.1 software and recorded as number of nucleotide bases (horizontal axis). The emission intensity differences between alleles and their "stutter bands" (McConnell et al. 1995) illustrate the level of discrimination afforded by this system and not possible by visual observation of the gel image.

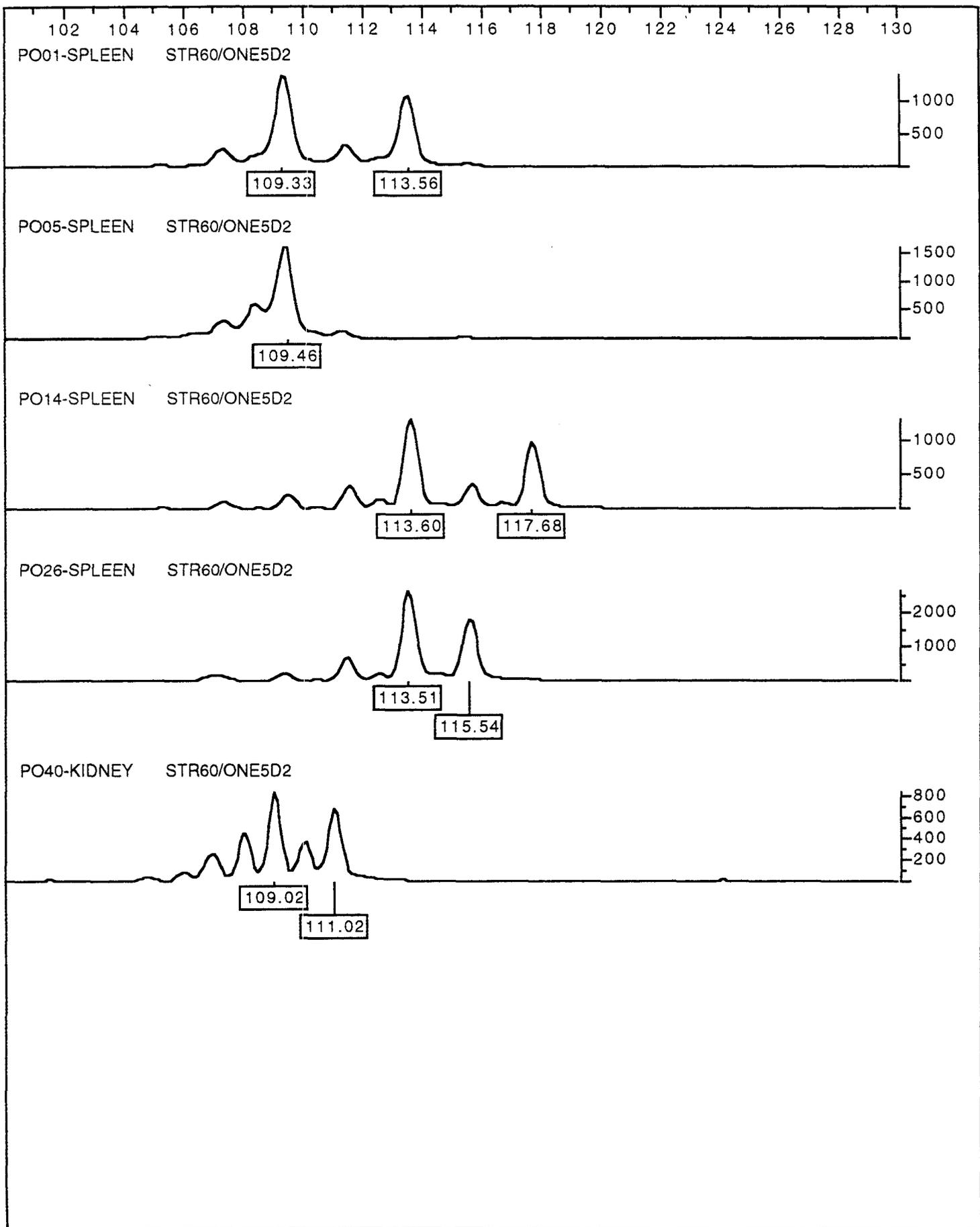
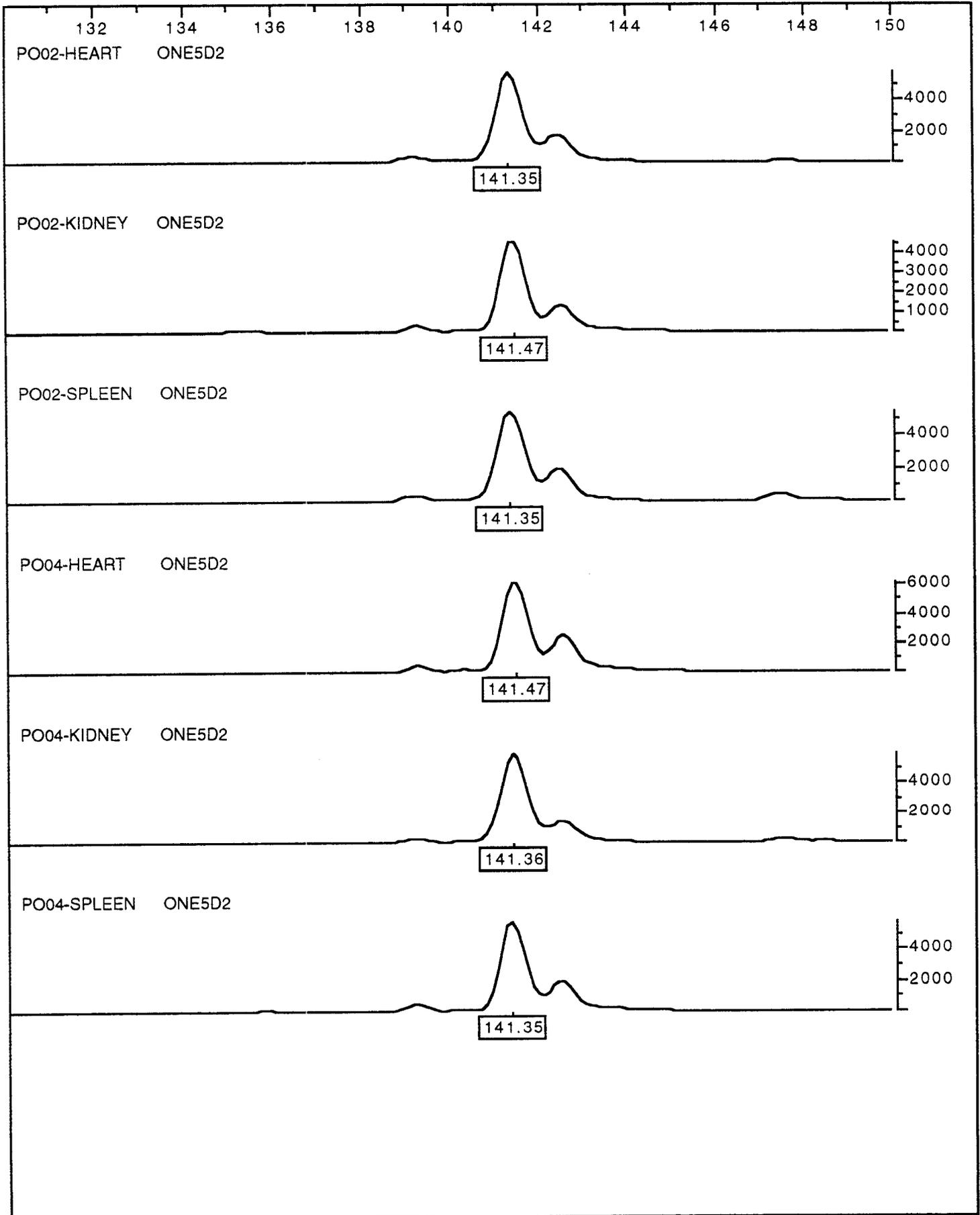


Figure 2. Electropherogram showing microsatellite locus *Oneu11* (*One5D2*) from three tissues (heart, liver, kidney) in two oiled pink salmon. No evidence of variation in heterozygosity was detected among 563 locus/tissue samples.





**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.**

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**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 Prudhoe Bay 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.

## 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  $\mu$ l with 200  $\mu$ M each dNTP, 10mM Tris, pH 8.3, 50 mM KCl, 7.5 pmol Hex dye-labeled *Tcl* 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 *Sma*I (unlabeled) primer (5' TAC CAA CTg AgC TAC AgA Agg ACC 3'; Kido et al., 1991), 2.0 mM MgCl<sub>2</sub>, 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  $\mu$ l with dH<sub>2</sub>O, and 4  $\mu$ l 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

*Tcl*/*Sma*I 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

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). *Tcl/Smal* 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 *Tcl/Smal* 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 *Tcl/Smal* fingerprinting for mutation screening is limited to scoring the presence or absence of segments of the genome which are amplified between adjacent *Smal* and *Tcl* sequences. In other words, *Tcl/Smal* 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 *Tcl/Smal* fingerprints is complicated in regions where comigrating amplified PCR products exist. As *Tcl/Smal* 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 *Tcl/Smal* fingerprinting screen. Both *Tcl/Smal* 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 *Tcl/Smal* fingerprinting. The resolution of fragments on minisatellite blots is plus/ minus 200-300 nucleotides, whereas resolution with *Tcl/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 *Tcl/Smal* 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 *Tcl/Smal* 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 *Tcl/Smal* 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.

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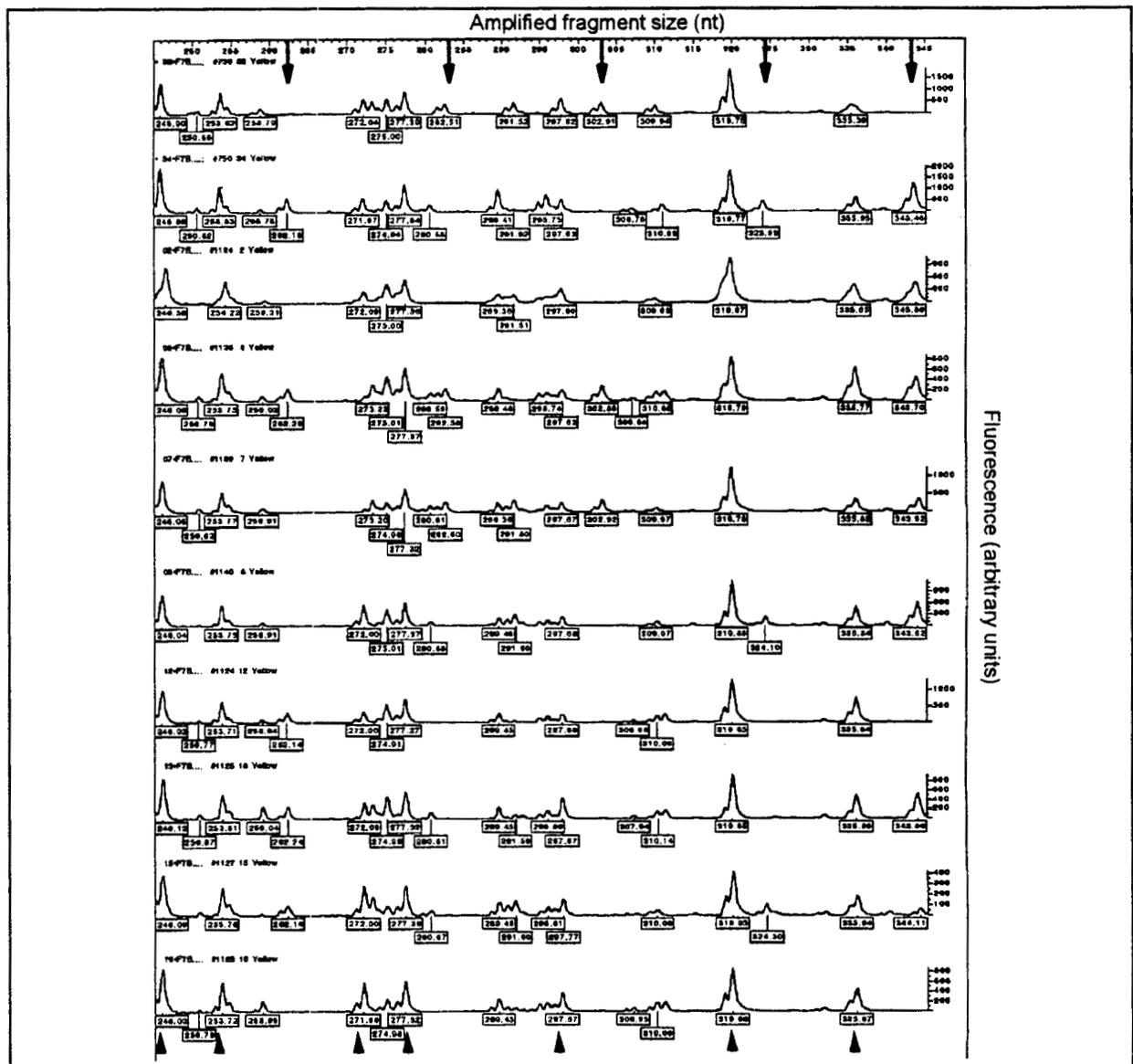
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**Figure 1.** Representative segment of aligned fingerprint electropherograms from 2 parental and 8 progeny DNAs of family 7B showing amplified loci in the range of 245-345 bases in length. The first two fingerprints, (#739 and 750) were from paternal 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.**

**(A) Summary of all loci scored**

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.

(A) Summary of all loci scored

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.

(A) Summary of all loci scored

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.**

**(A) Summary of all loci scored**

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.**

**(A) Summary of all loci scored**

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