

Construction of a Linkage Map for the Pink Salmon Genome

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ABSTRACT

This is the final year of our project based upon experiments conducted at the Alaska SeaLife Center (ASLC) that use the linkage map we have constructed to test for effects of regions of the genome on traits that are important to recovery of pink salmon (e.g., growth and survival). In summer 2001, we collected 259 sexually mature adults in Resurrection Bay from the 1999 cohort produced from wild pink salmon collected from Likes Creek. We will complete the analysis of the genotypes in the returning adults to test for genetic differences in marine survival and other life history traits (e.g., body size, egg number, and egg size).

INTRODUCTION

This is the final year of our research to construct a genetic linkage map for pink salmon (*Oncorhynchus gorbuscha*) and to use this map to evaluate effects of natural selection on the genome of this species. Such a map was proposed initially to provide the necessary platform to identify genetic damage in pink salmon inhabiting oiled streams following the March 1989 *Exxon Valdez* oil spill (EVOS). We have conducted a series of experiments based at the Alaska SeaLife Center (ASLC) to identify regions of the genome that affect various organismal traits and to test for the effects of natural selection on regions of the genome that include markers used to describe genetic population structure. This research will aid recovery efforts with pink salmon, including estimation of straying rates, description of stock structure, and testing if marine survival and other organismal measures of phenotypic variation have a genetic basis. We are asking for a reduced budget to complete our research.

Genetic linkage maps have provided the necessary information for understanding genetic variation in species since the rediscovery of Mendel's principles early in this century. A genetic map plays a similar role for a geneticist that a geographical map plays for the explorer of new territories. For many years, genetic maps could only be constructed in a very few model species that were suitable for extensive genetic manipulation (e.g., *Drosophila* and mice). Recent advances in molecular genetics now make it possible to uncover enough genetic markers to construct a detailed genetic linkage map in almost any species (Postlethwait et al. 1994).

This project began in FY 96. However, we did not receive authorization to proceed until half-way through FY 96 (March 1996). We have completed our two initial objectives that included identifying several hundred genetic markers and using them to construct a linkage map. We have used the ASLC for experiments that apply the linkage map to an understanding of the fundamental population biology and genetics of pink salmon.

This work was originally designed to support work with pink salmon under the project *OilRelated Embryo Mortalities* (Restoration Study \191A). The objective of that project was to identify germline mutations in pink salmon exposed to oil. Genetic damage induced by oil may either be small changes in nucleotide sequence (microlesions) or large-scale changes in chromosome structure (macrolesions). A detailed genetic map for pink salmon would have been invaluable for interpreting the results of Restoration Study \191A in several ways. First, it would be possible by following the inheritance of any DNA lesions to determine if they are micro- or macro-lesions. Second, these lesions could be mapped to determine if they are randomly spread throughout the genome or if they occur at mutational "hot spots" that are susceptible to oil induced damage. However, Restoration Study \191A is no longer ongoing, and thus our future work will concentrate on our original Objectives 5 and 6 as described in this proposal.

We propose to complete the analysis of our experiments at the ASLC to test for regions of the genome associated with marine survival and fitness. We collected 259 sexually

mature adults that returned to Resurrection Bay and the ASLC that were produced in 1999 from wild pink salmon collected from Likes Creek. We have identified the parents of these fish based upon genotypes at eight microsatellite loci and a growth hormone locus.

NEED FOR THE PROJECT

A. Statement of Problem

Elevated embryo mortalities were detected in populations of pink salmon inhabiting oiled streams following the spill. These increased rates of mortality persisted through the 1993 field season, three generations after the oil spill, suggesting that genetic damage may have occurred as a result of exposure to oil during early developmental life-stages. The consequences of the putative genetic damage include impaired physiological function of individuals and reduced reproductive capacity of pink salmon populations (Bue et al. 1998).

The aggregate of evidence from field studies and incubation experiments suggests that embryos exposed to oil in 1989 and 1990 accumulated deleterious mutations in the germline (Bue et al. 1998). However, see Cronin and Bickham (1998) for an alternative interpretation of these data. This hypothesis of genetic damage is consistent with previous field observations and laboratory experiments on the effects of crude oil on early life stages of fish. Long term intragravel oil exposures (78 months) to freshly fertilized eggs provide embryos sufficient time to accumulate polynuclear aromatic hydrocarbons (PAH's) from very low aqueous concentrations of crude oil. PAH's are abundant in crude oil and are potent clastogens (i.e. capable of breaking chromosomes). Roy et al. (1999) have recently reported evidence of molecular genetic damage to pink salmon embryos exposed to crude oil.

Mironov (1969) observed reduced survival of fish embryos and larvae exposed to very low aqueous doses (1 ul oil/L seawater) of oil. Longwell (1977) reported genetic damage in pelagic embryos affected by the ArgoMerchant oil spill. Moles et al. (1987) confirmed that pink salmon embryos take up PAH's and demonstrated that the uptake was much greater in an intertidal environment than in strictly freshwater conditions. Biggs et al. (1991) found greater numbers of chromosome aberrations in larval herring that incubated in oiled areas than in nonoiled areas. It is likely that the same type of damage may have occurred in pink salmon and other species in Prince William Sound, and this damage could have affected the germline of exposed individuals (Malkin 1994; Bue et al. 1998).

Molecular genetic techniques have been used extensively to describe population structure of Pacific salmon (Utter et al. 1993; Gharrett and Smoker 1994; Seeb et al. 1998). Genetic divergence among populations has been interpreted as largely reflecting the patterns of exchange of individuals among populations (gene flow) and random changes in frequency of selectively neutral alleles within populations (genetic drift) (Allendorf and Phelps 1981; Waples 1995). This is a useful approach that allows description of the pattern and amount of gene flow among populations.

This approach to describe population structure is based upon the assumption that the pattern and amount of divergence observed is not affected by natural selection or mutation. However, even weak natural selection may have a substantial effect on the pattern of genetic divergence among populations (Allendorf 1983). In addition, different mutation rates at marker loci may also effect the amount of genetic differentiation between populations, in particular if mutation rates at some loci are high (e.g., Jin and Chakraborty 1995). Thus, the high frequency of mutations that we have detected may also have a substantial effect on the amount and pattern of genetic divergence at some loci.

Molecular markers may be affected by natural selection even if the markers themselves are not the target of selection. Loci that are selectively neutral and have no effect on the phenotype are expected to be affected by the action of natural selection at closely linked loci (Slatkin 1995). Apparent heterozygous advantage ("associative overdominance") can result at neutral loci by linkage disequilibrium with nearby loci that are affected by natural selection (Pamilo and Pálsson 1998). Zhivotovsky et al. (1994) have recently questioned the description of genetic population structure of pink salmon and suggested that natural selection may have an important effect on allozyme frequency divergence in pink salmon.

It has been notoriously difficult to detect and measure the effects of natural selection in natural populations (Lewontin 1991). Comparing the distribution of genotypes in a single cohort followed through different life history stages is the most powerful method to detect natural selection (p. 303, Lynch and Walsh, in preparation). The facilities at the ASLC provide an exceptional opportunity to measure lifetime fitness from fertilization to sexual maturity of molecular genetic markers spread throughout the genome identified in previous years of this project.

B. Rationale/Link to Restoration

The recovery objective for pink salmon is healthy and productive populations that exist at prespill levels or levels in unoiled areas. An indication of recovery is when egg mortality in oiled areas match prespill or levels in unoiled areas. A genetic map would be essential for detecting and understanding causes of reduced egg and embryo survival in oiled areas (Bue et al. 1998). The genetic damage caused by exposure to oil may persist longer in populations of pink salmon than in other vertebrates because of the tetraploid nature of the salmonid genome. Salmonid fishes went through a tetraploid event some 25 million years ago that duplicated their entire genome (Allendorf and Thorgaard 1984). The extra genes in pink salmon may mask the effects of mutational damage caused by recessive deleterious alleles. The effects of these deleterious mutations may be uncovered in subsequent generations.

This research will provide a powerful test of the assumption of the absence of natural selection affecting molecular markers. This assumption is the foundation of interpreting patterns of genetic divergence among populations as reflecting patterns of genetic exchange. Evidence of natural selection affecting the molecular markers would cause a major change in the interpretation of genetic variation in natural populations of pink salmon

and other species. This will be true whether the selection is acting on the markers themselves or chromosomal segments linked to the markers. Recent results from molecular studies of the genome suggest that natural selection may play a greater role than previously thought in determining the structure of the genome, including the organization of genes and chromosomes, as well as the patterns and amounts of genetic variation present (Hurst 1999).

C. Location

Gametes for the inheritance studies and linkage map were collected from Prince William Sound in collaboration with the project Oil-Related Embryo Mortalities (Restoration Study \191A). Embryo incubation took place at the Genetics Lab facilities of ADFG. The laboratory analyses were done at the University of Montana and the ADFG genetics lab in Anchorage.

We began in FY 1998 to use the ASLC Research Facilities at Seward for experiments designed to test for natural selection at loci throughout the genome of pink salmon. Sexually mature pink salmon used in the experimental matings in 1998 and 1999 were collected from Likes Creek in Resurrection Bay. The progeny were marked with an adipose fin clip and released into Resurrection Bay. Due to the failure of the fish pass at the ASLC to attract returning adults we adjusted our plans for recapturing returning adults from the 1999 cohort to include sampling in upper Resurrection Bay.

COMMUNITY INVOLVEMENT

This is a specialized project that will not benefit directly from the knowledge of local/traditional people. We developed two computer interactive educational games that were incorporated into displays describing our project at the ASLC ("Lost Child" and "Whose Your Father?"). In addition, we have taken opportunities to explain our research in different Alaska high schools. Kathy Knudsen gave a presentation on this project to the freshman biology class at Kenai High School, and Kate Lindner discussed this study with high school students in Kongiganak.

We involved the community of Seward in helping us collect the returning adults. A lottery was conducted during the 2000 and 2001 field seasons as an incentive to recreational fisherman to turn in any marked pink salmon they caught. This resulted in 18 recreational fishermen turning in 22 marked fish in 2001 and 38 recreational fishermen turning in 61 marked fish in 2002.

PROJECT DESIGN

A. Objectives

Our initial primary objective was to construct a detailed genetic linkage map for pink salmon by analyzing the genetic transmission of several hundred DNA polymorphisms. Pink salmon have 26 pairs of chromosomes ($2N=52$; Allendorf and Thorgaard 1984), and, therefore, should have a total of 27 linkage-groups: 25 autosomes, an X-chromosome, and a Y-chromosome. We planned to map enough variable markers so that a new marker can be assigned with high probability to one of the 27 linkage groups. It was impossible to know how many markers this would require because we did not know the total length of the pink salmon linkage map. The linkage map of the zebrafish (*Danio rerio*) has been estimated to be 2900 centimorgans (cM; Johnson et al. 1996) and that of the medaka (*Oryzias latipes*) to be 2480 cM (Wada et al. 1995). There currently are efforts to include zebrafish among genome projects of model species sponsored by the National Institutes of Health under the Human Genome Project (Roush 1997). Such a massive effort in zebrafish would provide extremely helpful information for understanding the genome of salmonid fishes.

We expected the pink salmon map to be large because of the polyploid ancestry of salmonids and due to the fact it is female based. Young et al. (1998) recently have published a rainbow trout (*Oncorhynchus mykiss*) linkage map based upon recombination rates in males and estimated the total map to be 2628 cM. However, the linkage map in males will be shorter than in females because of the reduced recombination rate in male salmonids (Johnson et al. 1987a). We initially anticipated that it would be necessary to map over 500 markers to ensure that new markers can be assigned to an existing linkage group with high probability (Van der Beek and Van Arendonk 1993). For example, 99% of all loci in the zebrafish were estimated to be located within 20 cM of a marker on the map based upon an earlier report using 414 markers (Postlethwait et al. 1994).

This project originally had the following overall specific objectives:

1. Develop several hundred variable DNA markers in pink salmon and test them for Mendelian inheritance.
2. Construct a linkage map based upon joint segregation patterns of the DNA polymorphisms detected in previous objective.
3. Map putative lesions identified in Restoration Study \191A.
4. Test for Mendelian inheritance of markers throughout the genome in progeny of fish exposed to oil. Regions that show aberrant segregation ratios in progeny of fish exposed to oil and normal 1:1 ratios in fish not exposed to oil would be candidates for oil-induced lesions.
5. Test for regions of the genome that are associated with traits of adaptive significance (e.g., marine mortality or run-timing).

6. Test if protein markers (allozymes) are under natural selection such that they may not provide accurate information about the genetic structure and amount of gene flow among populations.

We have completed Objectives 1 and 2. We cannot pursue Objective 3 because Restoration Study /191A did not identify any putative lesions for mapping. At present, we do not intend to pursue Objective 4 because Restoration Study \191A is no longer ongoing. However, this type of experiment to detect oil-induced lesions could be pursued in the future at the ASLC. The primary focus in FY 03 will be completing Objectives 5 and 6; we propose to use the linkage map to test if there are phenotypic effects and adaptive significance of different classes of molecular markers.

B. Methods

OBJECTIVES 1 & 2

Our initial map was constructed using gynogenetic haploid and gynogenetic diploid progeny from an odd-year individual female (95-103). This is the same procedure that has been used to build the zebrafish linkage map (Postlethwait et al. 1994). Stanley (1983) reported that haploid embryos of Atlantic salmon will develop until just prior to the stage of hatching if development of the eggs is activated by sperm in which the DNA has been inactivated by UVradiation. We have used this technique routinely with fishes of the genus *Oncorhynchus* (Forbes et al. 1994; Spruell et al. 1999). This allows us to follow the segregation and linkage relationships in haploid progeny from females. The use of haploid progeny avoids possible difficulties of dominance with some types of DNA markers because recessive alleles are not obscured by their dominant alternatives in haploids (Lie et al. 1994). Our odd-year map is primarily based on 603 segregating markers in 94 haploid progeny from a single pink salmon female (A95-103) that returned to Armin F. Koernig hatchery in Prince William Sound in August 1995. We have placed a number of so-called "anchor" loci on this map.

In addition we have completed the construction of a linkage map based on the segregation pattern of 90 haploid individuals in an even-year female (V96-13). Odd- and even-year pink salmon are reproductively isolated due to the fixed two-year life cycle of this species (Aspinwall 1974). Beacham et al. (1988) report substantial allozyme and morphological evidence for differentiation of alternate brood years. In addition, Phillips and Kapuscinski (1988) and Phillips et al. (1999) detected chromosomal rearrangements between odd- and even-year populations that occur in the same geographical area. Furthermore, in a recent experimental study, Gharrett et al. (1999) demonstrated outbreeding depression in crosses between the two year classes. Together, these finding suggest that the alternate brood years are reproductively isolated and genetically distinct. Having linkage data from both odd- and even-year individuals will make it possible to map more markers and will allow us to determine whether linkage relationships are conserved between the reproductively isolated year classes.

Differences in meiosis between male and female salmonids have been found in all species that have been examined (Allendorf and Thorgaard 1984; Johnson et al. 1987a). There generally is greater recombination in females than in males (Johnson et al. 1987a; Allendorf et al. 1994). In addition, only disomic inheritance has been reported in females. However, in males some loci show patterns of segregation that approach those expected with tetrasomic inheritance (Allendorf and Thorgaard 1984).

Construction of a full linkage map is a large task. We developed as many time and labor saving procedures as possible. Our linkage map was constructed by computer assisted analysis (MapMaker, Lander et al. 1987). We have been assisted by Mark Daly of the Whitehead Institute at MIT in using this program. We will compare the recombination rates based upon this map to rates of selected pairs of loci in males using families produced from the 1998 cohort. The reduced recombination rates in salmonid males means that it will be easier to assign new markers to a linkage group using male parents. We will test joint segregation of individual markers from different linkage groups identified in females to determine if some of these separate linkage groups in females are linked in males and are therefore syntenic (on the same chromosome).

A useful genetic map contains genetic markers that are abundant, randomly distributed throughout the genome, highly polymorphic, and readily detectable in many laboratories (Jacob et al. 1995). We began using random amplified polymorphic DNA (RAPD) markers because they fit these criteria and they have been used successfully in constructing linkage maps in zebrafish and medaka (Johnson et al. 1996; Wada et al. 1995). We have switched to two other types of genetic markers that are superior to RAPDs in this work.

PINES: There are a variety of repetitive DNA elements that are scattered throughout the genome of salmonid fishes. Greene and Seeb (1997) have described a technique that uses the sequences from a SINE (short interspersed nuclear element) and a transposon to detect many DNA polymorphisms. They have called this technique SINEprinting. We have modified this technique using other types of repetitive elements for our mapping study to detect a class of molecular markers that we call PINES (paired interspersed nuclear elements; Spruell et al. 1999).

Kido et al. (1991) described 3 SINES in salmonid fishes. They documented the presence of two such elements, *HpaI* and *SmaI*, in pink salmon. Spruell and Thorgaard (1996) subsequently reported the presence of the 5'-end of the third element, *FokI*, in pink salmon. Goodier and Davidson (1994) confirmed that salmonids also contain the transposon *Tc1*, a member of another class of repetitive elements. Both SINES and transposons occur in high copy number and are believed to be ubiquitously dispersed throughout the genome, making them ideal candidates for genomic mapping efforts.

We have used DNA sequences from four types of repetitive elements as polymerase chain reaction (PCR) primers to generate multiple DNA fragments from a single PCR reaction in pink salmon. The theoretical basis for this procedure is similar to the use of the human SINE *AluI* to identify human chromosomes in somatic cell hybridization

experiments (Nelson et al. 1989). Primers complementary to one end of the element are oriented such that they initiate DNA synthesis from the end of the element, progressing into the surrounding genomic DNA. A single primer or combinations of primers may be used to generate multilocus patterns. Greene and Seeb (1997) used this technique to confirm the parentage of pink salmon fry, demonstrating the potential utility of including these fragments in our mapping study. We have used 12 different pairs of PINE primers to detect 162 segregating markers in our reference family.

AFLPs: Amplification fragment length polymorphisms have been used extensively in the construction of genomic maps in plants (Maheswaran et al. 1997; Becker et al. 1995). The AFLP technique is especially advantageous for two reasons. First, many bands are produced per reaction and, therefore, more polymorphic loci are produced per unit effort. Second, the selective amplification step uses a subsample of the PCR products of the preamplification. Up to 133 selective amplifications can be completed from a single pre-amplification that originally used only 0.5 µg of genomic DNA. Much less genomic DNA is needed to produce more bands than using other methods such as RAPDs. This is an important consideration when dealing with the limited amount of tissue available from haploid embryos.

Gene-Centromere Map

We estimated recombination rates between 312 loci and their centromeres using half-tetrad analysis in a recently published manuscript (Lindner et al. 2000). We produced the half-tetrads by initiating development with irradiated sperm and blocking the maternal second meiotic division. AFLPs were significantly more centromeric than loci identified by three other techniques (allozymes, microsatellites, and PINES). The near absence of AFLPs in distal regions could limit their utility in constructing linkage maps. A large proportion of loci had y values approaching 1.0, indicating near complete crossover interference on many chromosome arms. As predicted from models of chromosomal evolution in salmonids, all duplicated microsatellite loci that shared alleles (isoloci) had y values of nearly 1.0. This is consistent with previous data from allozyme loci.

Odd-Year Linkage Map

We have described the segregation of 590 markers in haploid progeny from female A95-103; we have also mapped 13 allozyme loci in the same female. We assigned 546 of the 590 DNA markers and all of the allozyme loci to one of 44 linkage groups covering a distance of 4559 cM. Given the haploid number of 26 chromosomes for pink salmon, our mapping efforts produced 18 extra linkage groups. Taking into account the extra linkage groups and 44 unassigned markers as well as the distance to the telomeres we estimate the size of the pink salmon genome to be 6691 cM. The haploid pink salmon genome is approximately 2.72 million kilobase pairs (kpb; Johnson et al 1987b); thus, we estimate approximately 406 kbp/cM. These results are consistent with our expectations when comparing to maps constructed in other fishes.

Even-Year Linkage Map

We have completed construction of a linkage map for even-year pink salmon from Prince William Sound. We have analyzed the segregation pattern of 85 loci in an even-year family (V96-13) and assigned 63 of 85 loci to one of 22 linkage groups. One gene of known function, *MHCB 2*, is assigned to a linkage group that consists of one microsatellite and two PINE loci.

We have not found any differences in location of loci on the maps or recombination rates between the odd- and even-year maps. We are completing the comparative analysis of the odd- and even-year maps, and will submit a manuscript for publication within three months.

Mutation Analysis

Our results have provided exciting and important information about mutation processes in microsatellites that are accepted for publication in the journal *Molecular Biology and Evolution* (Steinberg et al. 2002). Our experimental design depends upon being able to place returning adults into their correct family on the basis of their multiple-locus genotypes. We tested this by examining inheritance data at 11 loci (nine microsatellites and two genes of known function) for 10 progeny from each of the 50 families that were released in spring of 1999. In the process of analyzing the inheritance data, we detected several mutations at two of the microsatellite loci (*SSA408* and *OGO1c*), indicating that these loci have particularly high mutation rates. Furthermore, at *SSA408* the mutations detected were not distributed randomly among families. Rather, clusters of identical mutant alleles were found in certain families, suggesting they may have resulted from mutation events occurring very early in gametogenesis, prior to meiosis.

Our results have important significance for the use of microsatellite loci in management. Mutations are expected to have a substantial effect on the amount and pattern of genetic divergence among populations if the mutation rate approaches the rate of migration among populations (see discussion in Allendorf and Seeb 2000). Not surprisingly, the number of mutations detected was correlated with the number of alleles in the sample (Table 8). We detected mutations at the two loci that have the greatest number of alleles in the parental population (*OGO1c* and *SSA408*). The mutation rate estimates at *OGO1c* and *SSA408* (3.7×10^{-3} and 5.4×10^{-3}) are at the high end of the range of 10^{-3} to 10^{-6} reported for other organisms (Dallas 1992, Weber and Wong 1993, Schug et al. 1997). The variability of these two loci makes them powerful tools for assigning parentage. We were able to unambiguously assign parentage to 35 of the 36 returning fish from the 1998 cohort based on these two loci alone. However, given the high probability of mutation at these loci, our results indicate that it is important to use a combination of low and high variability markers for parentage analysis. Our mutation analysis also suggests that *OGO1c* and *SSA408* are inappropriate as markers for analysis of stock structure in pink salmon.

OBJECTIVES 5 & 6

The completion of a genome map for pink salmon provides important information for addressing genetic issues related to two other Components of the Pink Salmon Restoration Program. The numerous genetic markers identified in the course of this study will provide greatly increased power and resolution to identify stocks of pink salmon on a very fine scale (Stock Separation and Management). In addition, understanding the process of mutation will help identify appropriate markers for use in stock identification. The genome map also provides a platform to test for the presence of genes having major effects on traits of importance for the management of pink salmon, and to test for phenotypes associated with specific combinations of multilocus genotypes (Lander and Schork 1994). These genetic markers will be of great value in identifying fish from supplementation programs (hatchery-raised stocks) and detecting their ecological and genetic interactions with wild fish (Supplementation).

This aspect of the research was being performed at the ASLC research facilities. Approximately 50,000 and 24,000 marked fish were released in spring of 1999 and 2000 respectively. We collected 36 sexually mature adults in Resurrection Bay from the 1998 cohort and 259 sexually mature adults in Resurrection Bay from the 1999 cohort produced from wild pink salmon collected from Likes Creek. A sample of the fish was collected at release and will be analyzed so that their genetic characteristics prior to the marine phase of the life cycle can be compared to the returning adults. We will test for genetic effects on phenotypes of special importance by comparing the genotypes of the released fish with the genotypes of the returning fish. This will allow us to test for genes with a major effect on marine survival. We will test for loci or regions of the genome that have a large effect on phenotypes of interest, so-called quantitative trait loci (QTL's). For example, Jackson et al. (1998) recently have presented evidence for QTL's that affect upper temperature tolerance in rainbow trout linked to two of 24 polymorphic loci that they examined. Mousseau et al. (1998) have used a similar approach to estimate heritabilities for weight, length, and age at sexual maturation in chinook salmon.

Previous work has demonstrated genetic differences in run-timing has a genetic basis (McGregor et al. 1998; Smoker et al. manuscript). We will compare the genotypes of fish returning to the facility at different times to test for genes with a major effect on run timing. We will use a suite of genetic markers spread uniformly throughout the genome. Regions of the genome that show major associations with run-timing can then be examined in more detail by comparing additional markers within that region. A similar approach using only 10 protein markers in hatchery rainbow trout revealed several regions of the genome associated with time of spawning (Leary et al. 1989). Sakamoto et al. (1999) have reported similar results on the basis of 54 microsatellite loci.

Karl and Avise (1992) reported concordant patterns of genetic differentiation for mitochondrial DNA and four nuclear DNA loci in the American oyster (*Crassostrea virginica*) along the east coast of North America. In contrast, previous allozyme studies had not detected these genetic differences among these same populations. Karl and Avise

concluded that the pattern observed for the DNA markers reflected the historical patterns of isolation and gene flow among these populations while this pattern is obscured in the allozymes because of "balancing selection" at the allozyme loci. Similar results have been reported in the Atlantic cod (Pogson et al. 1995). These results provide an important challenge to the generally accepted utility of allozyme markers for describing historical patterns and amounts of gene flow between populations. That is, if allozymes are under strong natural selection then they may not provide accurate information about the genetic structure and amount of gene flow among populations.

Restoration Projects 95320D and 96196 have described the genetic population structure in Prince William Sound (PWS) odd- and even-year fish at allozyme loci and mitochondrial DNA (mtDNA) (Seeb et al 1996; Habicht et al. 1998). These studies reported small but statistically significant genetic allele frequency differences among streams, and concluded that pink salmon in PWS should be managed taking into account subpopulation structure rather than as a single panmictic population. As is usually done in such studies, these authors assumed that the genes they examined were selectively neutral (that is, not affected by natural selection). However, the estimates of these authors could be severe overestimates of the actual amount of gene flow if "balancing" selection is maintaining similar frequencies (Karl and Avise 1992; Pogson et al. 1995). That is, there may be much less gene flow among populations than is suggested by these studies.

Zhivotovsky et al. (1994) have reviewed population genetic data of pink salmon and concluded that the interpretations concerning amounts and patterns of gene flow are questionable because even weak natural selection could have a major effect on genetic divergence among populations of pink salmon. A series of papers by Altukhov and his colleagues has provided evidence for phenotypic and fitness effects of genetic variation at allozyme loci in pink salmon (Altukhov 1990; Altukhov et al. 1987, 1989; Dubrova et al. 1995; Kartavtsev 1992). These papers argue that genotypes at allozyme loci have a significant effect on marine survival, growth rate, and several other important factors.

The clearest and perhaps most important effects have been demonstrated on marine survival and growth rates. Pink salmon that are more heterozygous at allozyme loci have greater viability and growth rates than more homozygous individuals (Altukhov et al. 1991; Zhivotovsky et al. 1987; Kartavtsev 1992). Table 1 shows the distribution of individual heterozygosities at four allozyme loci in fry before release into salt water and returning adult spawners in odd-year pink salmon from the Sakhalin Island (Altukhov et al. 1987). We would expect the heterozygosities in fry and adults to be similar if the genotypes at these loci are not associated with survival. The significantly higher heterozygosity in the returning adults (0.619) than in the fry (0.424) indicates that individuals that were more heterozygous at the four loci had greater marine survival.

Altukhov et al. (1991) found a significant positive regression ($r=0.14$; $P<0.01$) between individual heterozygosity at these same four allozyme loci and body length of fry immediately preceding downstream migration from a hatchery on the Sakhalin Island. Kartavtsev (1992) reported a similar relationship in a different experiment with pink salmon

from Sakhalin island ($r=0.23$; $P<0.001$). Previous studies with salmonids have found that size has an important effect on survival (Hunt 1969).

Table 1. Distribution of Heterozygosity at Four Allozyme Loci in Pink Salmon from Sakhalin Island

$\chi^2 = 37.3$
d.f. = 2

* values are the frequencies (and number) of individuals with the indicated number of heterozygous loci.

Similar results have been reported in other salmonid species for many phenotypes of evolutionary importance (e.g., developmental rate, egg size, and disease resistance; reviewed by Ferguson 1992). Positive associations between heterozygosity at allozyme loci and important phenotypic characters, such as growth rate, survival, fertility, disease resistance, developmental rate, and developmental stability, have been described in many organisms (reviewed by Zouros and Foltz 1986; Allendorf and Leary 1986).

The mechanism underlying these associations remains unknown. The most likely explanations are (1) the associations are the consequence of heterozygosity at the loci examined, or (2) the loci examined may be in linkage disequilibrium with other loci that affect the traits being studied (associative overdominance; Leary et al. 1987).

It has been argued that these relationships between multiple locus heterozygosity and phenotypes have been found with allozymes because these loci are important in ATP production and protein catabolism (Koehn et al. 1988). We propose to distinguish between these hypotheses by using the linkage map to compare the effects of different markers on marine survival and other traits. If the enzyme loci themselves are responsible for this effect, then we would expect to find an association between enzyme genotypes and survival, but not between genotypes at DNA markers spread throughout the nuclear genome. However, if we find a similar association using DNA markers, this would suggest that the effect is due to chromosomal segments and not the enzyme loci themselves.

We believe that it is unlikely that the enzyme loci themselves are responsible for the observed relationships. Nevertheless, regardless of the underlying mechanisms of these associations, even weak heterozygous advantage (or associative overdominance) would act to maintain similar allele frequencies in different populations in the absence of significant gene flow (Allendorf 1983). This could cause a large overestimation of the actual amount of gene flow among Prince William Sound pink salmon populations. For example, just a 10% selective advantage of heterozygotes will cause a 10fold over estimation of the amount of migration in the case where local populations have an effective size of 100 and an average 0.5 migrants per generation (Allendorf 1983). Altukhov et al. (1987) have estimated an average selective advantage of approximately 25% at four allozyme loci in pink salmon.

We will ask a series of questions in this aspect of the research. The primary question is are there regions of the genome that have a significant effect on survival during the marine phase of the life cycle? Secondly, we will ask if allozyme markers tend to occur in those regions that affect survival. We will also determine if selection favors heterozygotes.

Marine Survival and Fitness Experiment: 1998 cohort

In August 1998, 150 (75 male and 75 female) mature pink salmon were collected from Likes Creek, Resurrection Bay, and transported to the ASLC for controlled matings. We made 75 families of full-sibs by crossing one male and one female. One hundred progeny from each family were collected to test marker inheritance for parentage analysis. We then selected 50 of these families on the basis of egg number and survival during incubation for the release experiment. These families were pooled together into a single tank in March shortly after hatching. In May 1999, approximately 1,500 progeny from each of these 50 single-pair mating families were marked and released from the ASLC facility.

Progeny from this experiment returned in August 2000. We had anticipated a return rate of 2%, for a total of 1,000-2,000 individuals expected to be recovered for genetic and morphological analyses (approximately 30 fish per family). However, no fish returned to the ASLC fish pass, and we captured a total of 36 fish throughout Resurrection Bay. These 36 fish were placed into 30 families on the basis of 10 microsatellite loci. This sample size was too small to answer the questions that we are addressing.

Marine Survival and Fitness Experiment: 1999 cohort

t with odd-year pink salmon in August 1999. We collected 68 adults (34 females and 34 males) from Likes Creek, and released their marked progeny from the ASLC in May 2000. This cohort returned in the summer of 2001. We used a different experimental mating scheme with these fish to allow a more powerful genetic analysis of the progeny. Each male and each female was crossed with two individuals in a series of 2 x 2 diallel crosses (Figure 1).

[FIGURE 1 in WORD file (fig-1.doc)]

We collected 259 sexually mature adults from this cohort that returned to Resurrection Bay in summer 2001 (Figure 2). We have identified the parents of these fish based upon genotypes at eight microsatellite loci and a growth hormone locus. The distribution of returning progeny (i.e., reproductive success) of the 34 males and females is close to expected with random reproductive success (Table 2; Figures 3). The only apparent exception is that there are 2 males that each produced an exceptionally large number of returning progeny (18).

[TABLE 2 in WORD file (table-2.doc)]

[FIGURE 2 in WORD file (fig-2.doc)]

[FIGURE 3 in WORD file (fig-3.doc)]

We have begun analysis of the heritability of several morphological traits on the basis of parent-offspring regression from these fish. Heritability estimates of body length at sexual maturity (length) are surprisingly high. We did these separately for the sexes because males tend to be longer than females. The heritability of length in males is 0.45 (Figure 4, $P < 0.005$); the heritability of length in females is 0.34 (Figure 4, $P < 0.005$).

[FIGURE 4 in WORD file (fig-4.doc)]

We also analyzed the heritability of several components of female reproductive success: mean egg size, total egg number, and total egg weight. As expected, these traits that are closely related to fitness had low heritabilities in comparison as morphological trait such as body length. The heritability of mean egg weight was 0.32 ($P < 0.10$; Fig. 5). The heritabilities of total egg number and total egg weight were zero (Figure 6).

[FIGURE 5 in WORD file (fig-5.doc)]

[FIGURE 6 in WORD file (fig-5.doc)]

C. Cooperating Agencies, Contracts, and Other Agency Assistance

The ADFG Genetics Lab is no longer funded to assist us in the work at the ASLC. Therefore, we are currently doing all of the allozyme analysis at the University of Montana.

SCHEDULE

A. Measurable Project Tasks for FY 03 (1 October 2002 - 30 September 2003)

- 1 Oct - 31 Jan: Complete genetic analyses of returning adults from 1999 cohort. Initiate data analysis to test for correlations between markers from the linkage map and traits associated with marine survival and fitness in the returns of the 1999 cohort.
- 1 Dec - 30 Sep: Complete data analysis to test for correlations between markers from the linkage map and traits associated with marine survival and fitness in the returns of the 1999 cohort.
- 1 Jan - 30 Sep: Prepare manuscripts for publication describing results of marine survival and fitness experiment.

B. Project Milestones and Endpoints

- Objective 1: This objective has been completed.
- Objective 2: This objective has been completed.
- Objective 3: This objective will not be pursued.
- Objective 4: This objective will not be pursued.
- Objective 5: This objective will be completed by the end of year 8.
- Objective 6: This objective will be completed by the end of year 8.

C. Completion Date

We initially proposed to continue this work for five years. However, our release experiments were delayed until the ASLC facilities were available. The 1998 cohort fish released in the spring of 1999 returned at the end of year five, and the 1999 cohort fish returned at the end of year six. Genetic analysis will be completed in the first three months of year eight, and data analysis and will be completed by the end of year eight.

PUBLICATIONS AND REPORTS

Allendorf, F. W., P. Spruell, K. L. Knudsen, K. R. Lindner and K. L. Pilgrim. 1997. Construction of a Linkage Map for the Pink Salmon Genome, *Exxon Valdez* Oil Spill

Restoration Project Annual Report (Restoration Project 97190), University of Montana, Missoula, Montana.

Allendorf, F. W., P. Spruell, K. L. Knudsen, K. R. Lindner, D.J. Reedy, and K. L. Pilgrim. 1998. Construction of a Linkage Map for the Pink Salmon Genome, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 98190), University of Montana, Missoula, Montana.

Allendorf, F. W., P. Spruell, K. L. Knudsen, and K. R. Lindner. 1999. Construction of a Linkage Map for the Pink Salmon Genome, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 99190), University of Montana, Missoula, Montana.

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

Lindner, K. R., E. K. Steinberg, P. Spruell, K. L. Knudsen, A. Maxwell, and Allendorf, F. W. 2000. Construction of a Linkage Map for the Pink Salmon Genome, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 00190), University of Montana, Missoula, Montana.

Lindner, K.R., J. E. Seeb, C. Habicht, K.L. Knudsen, E. Kretschmer, D. J. Reedy, P. Spruell, and F. W. Allendorf. 2000. Gene-centromere mapping of 312 loci in pink salmon by half-tetrad analysis. *Genome* 43:538-549.

Lindner, K. R., E. K. Steinberg, M. Skinner, K. L. Knudsen, P. Spruell, and Allendorf, F. W. 2001. Construction of a Linkage Map for the Pink Salmon Genome, *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 01190), University of Montana, Missoula, Montana.

Steinberg, E.K., K.R. Lindner, J. Gallea, J. Meng, A. Maxwell, and F.W. Allendorf. 2002. Rates and patterns of microsatellite mutations in pink salmon. *Molecular Biology and Evolution* 19:1198-1202.

Lindner, K.R., P. Spruell, C. Habicht, J. E. Seeb, H. Zhao, and F. W. Allendorf. In preparation. Estimation of chiasma interference and construction of a linkage map for pink salmon. To be submitted to *Genetics*.

PROFESSIONAL CONFERENCES

We anticipate presenting our results at professional and scientific meetings.

COORDINATION AND INTEGRATION OF RESTORATION EFFORT

This work has been done in collaboration with James E. Seeb, Principal Geneticist, ADFG. The inheritance experiments were performed in coordination with the project OilRelated Embryo Mortalities (Restoration Study \191A). Dr. Seeb is no longer funded to collaborate with us in this Restoration Study.

EXPLANATION OF CHANGES IN CONTINUING PROJECTS

The changes in this proposal reflect the discontinuation of Restoration Study \191A, and the decision not to fund our ADFG collaborators on this project. We made changes in our plans to capture fish returning to Resurrection Bay based upon the lack of any returns to the ASLC in summer 2000.

PROPOSED PRINCIPAL INVESTIGATOR

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PRINCIPLE INVESTIGATOR

FRED W. ALLENDORF: Principal Investigator

BIRTH: 29 April 1947; Philadelphia, Pennsylvania

MILITARY SERVICE: U.S. Army, 1965-1968 (Vietnam, 1966-1967)

EDUCATION: B.S., Zoology, Pennsylvania State University, 1971
M.S., Fisheries, University of Washington, 1973
Ph.D., Genetics and Fisheries, University of Washington,
1975

POSITIONS:

1975-1976 Lektor, Department of Genetics and Ecology, Aarhus University, Denmark
1976-1979 Assistant Professor of Zoology, University of Montana
1978-1979 NATO Fellow, Genetics Research Unit, University of Nottingham,
1979-1984 Associate Professor of Zoology, University of Montana
1983-1984 Visiting Scientist, Department of Genetics, Univ. of California, Davis
1984-1989 Professor of Zoology, University of Montana
1989-1990 Program Director, Population Biology and Physiological Ecology,
National Science Foundation (NSF)
1992-1993 Visiting Professor, University of Oregon
2000-2001 Fulbright Senior Scholar, Victoria University of
Wellington, New Zealand
1990 Professor of Biology, University of Montana

HONORS: NATO/NSF Postdoctoral Fellowship, University of Nottingham, 1978-1979
European Molecular Biology Organisation (EMBO), Fellowship, University of
Stockholm, 1979
Distinguished Scholar Award, University of Montana, June 1985
Burlington Northern Faculty Achievement Award for Research, University of
Montana, June 1987
Elected Fellow, American Association for the Advancement of Science
(AAAS), February 1987
Burlington Northern Faculty Achievement Award for Research, University of
Montana, May 1991
Elected Member, AAAS Council (Biological Sciences Division),
1996-1998
President, American Genetic Association, 1997
Fulbright Senior Scholar Award, New Zealand, 2000

MAJOR GRANTS:

National Science Foundation Research Grant, EPSCR, 1980-1983, \$70,000
National Science Foundation Research Grant, Population Biology, 1980-1982,
\$60,000
National Science Foundation Research Grant, 1983-1986, \$121,000
National Science Foundation, Faculty Research Opportunity Award, 1986, \$10,000
United States Department of Agriculture Grant, Aquaculture, 1983-1985, \$43,000
National Science Foundation Research Grant, 1986-1989, \$148,000
National Science Foundation, Dissertation Research Grant, 1988-1990, \$9,850
National Science Foundation Research Grant, 1989-1993, \$150,000
National Science Foundation Research Grant, Conservation and Restoration Biology,
1993-1998, \$270,000
National Science Foundation Research Grant, Small Grant for Experimental Research,
Population Biology, 2000-2001, \$50,000

ASSOCIATE EDITORSHIPS: Evolution (1987-1990)
Journal of Heredity (1986-1989)
Progressive Fish Culturist (1986-1989)
Molecular Biology and Evolution (1994-1996)

EDITORIAL BOARDS: Molecular Biology and Evolution (1983-1989)
Conservation Biology (1990-1993)
Molecular Ecology (1991-present)

PROFESSIONAL SERVICE:

Panel Member, Population Biology and Physiological Ecology, NSF (1987-1989)
Panel Member, International Program, National Science Foundation (1987)
Panel Member, Conservation and Restoration Biology, NSF (1991-1992; 1995)
Council Member, The American Genetic Association (1986-1989)
Genetics Nomenclature Committee, American Fisheries Society (1986-present)
Member, Committee on the Protection and Management of Pacific Northwest
Anadromous Salmonids, National Research Council (1992-present)
Chair, Committee of Visitors, Systematic and Population Biology Programs, NSF
(1993)

PROFESSIONAL SOCIETIES: Society for the Study of Evolution
American Society of Naturalists
Genetics Society of America
Society for Conservation Biology
American Association for the Advancement of Science
American Society of Ichthyologists and Herpetologists
American Fisheries Society
American Genetic Association
Desert Fishes Council
Ecological Society of America

Montana Native Plant Society
Society of Systematic Biologists
Society for Molecular Biology and Evolution

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- Harris, R.B., W.A. Wall, and F.W. Allendorf. Submitted. Genetic consequences of hunting: what do we know and what should we do? *Wildlife Society Bulletin*.
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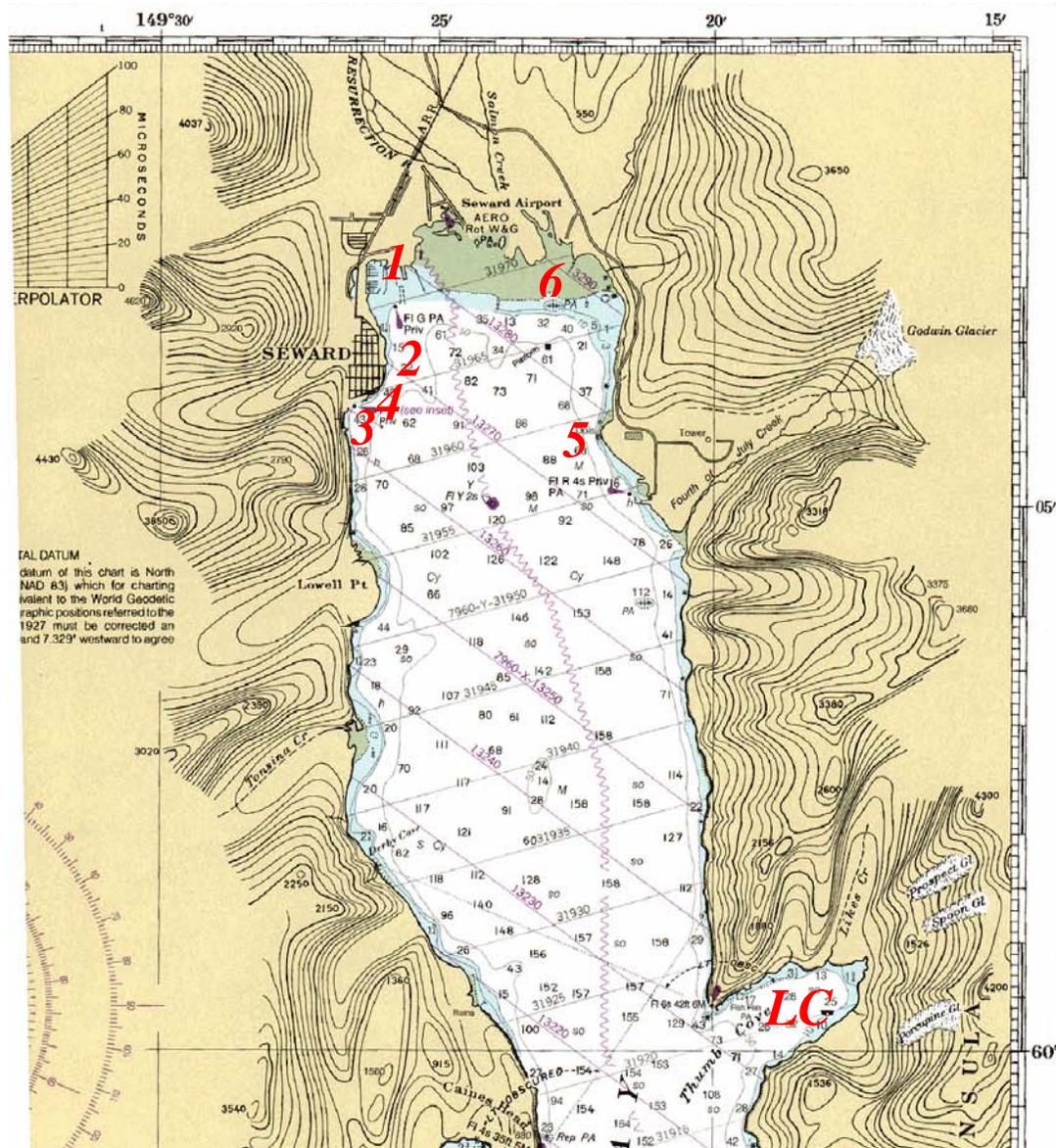
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Figure 1. Diagram of our half-sib family experimental design. Numbers across the top represent females, numbers down the side represent males. The squares contain the family designation and the numbers of individuals used to make each family. The number of the dam is the family number in each half-sibling cross. The letter A or B for each family designates the sire. The parents of family 5A are dam 5 and sire 105, 5B are dam 5 and sire 106, 6A dam 6 and sire 105, and 6B are dam 6 and sire 106, and so forth.

		FEMALES									
		01	02	03	04	05	06	...	33	34	
MALES	101	1A 01x101	2A 02x101								
	102	1B 01x102	2B 02x102								
	103			3A 03x103	4A 04x103						
	104			3B 03x104	4B 04x104						
	105					5A 05x105	6A 06x105				
	106					5B 05x106	6B 06x106				
	.								.		
	.								.		
	.								.		
	133									33A 33x133	34A 34x133
	134									33B 33x134	34B 34x134

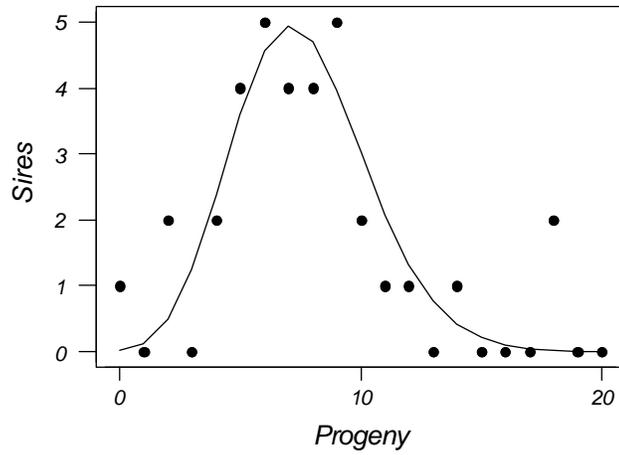
Figure 2. Map of Resurrection Bay. Numbers indicate the location where experimental fish were collected in 2001 as designated below. LC designates Likes Creek, the location where the parents were collected.



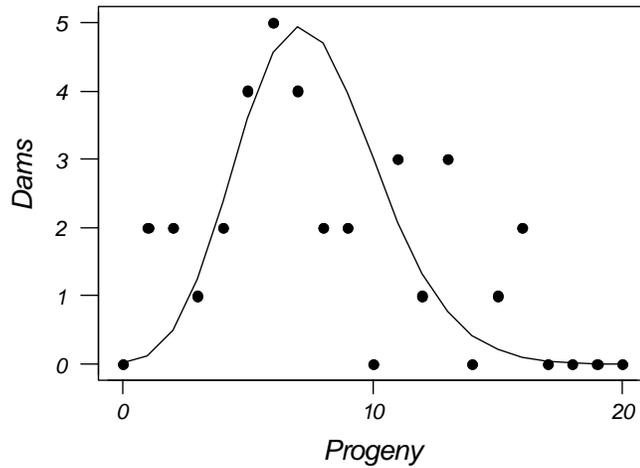
Map Number	Location	Number of fish collected
1	Boat Harbor	2
2	Culvert	31
3	Lowell Cr.	26
4	ASLC	182
5	Spring Cr.	4
6	Resurrection R.	10
	Unknown	4
	Total	259

Figure 3. Scatterplot of the number of progeny returning for sires (a) and dams (b). For example, there were two sires that produced 10 returning progeny. The line is the expected Poisson distribution.

(a)



(b)



FY 03 EXXON VALDEZ TRUSTEE COUNCIL PROJECT BUDGET

October 1, 2002 - September 30, 2003

Budget Category:	Authorized FY 02	Proposed FY 03					
Personnel	\$93.7	\$35.6					
Travel	\$1.8	\$1.5					
Contractual	\$0.0	\$0.0					
Commodities	\$29.7	\$1.2					
Equipment	\$0.0	\$0.0	LONG RANGE FUNDING REQUIREMENTS				
Subtotal	\$125.2	\$38.3	Estimated				
Indirect	\$31.8	\$11.7	FY 04				
Project Total	\$157.0	\$50.0	\$0.0				
Full-time Equivalents (FTE)		0.6					
Dollar amounts are shown in thousands of dollars.							
Other Resources							
<p>Comments:</p> <p>Indirect costs are based on the University of Montana rate of 43.7% of salaries and wages.</p> <p>Travel costs are included to attend the Trustee council Annual EVOS Workshop.</p> <p>Travel costs are included to attend one scientific meeting.</p> <p>Personnel time includes time for the final report and manuscript preparation.</p> <p>NOTE: ADF&G GA of 9% (\$4,500) needs to be added to this project, for total of \$54,500.</p>							

FY03

Prepared: July, 2002

Project Number: 03190
 Project Title: Construction of a Linkage Map for the Pink Salmon Genome
 Name: University of Montana

FY 03 EXXON VALDEZ TRUSTEE COUNCIL PROJECT BUDGET

October 1, 2002 - September 30, 2003

Contractual Costs:		
Description		
		Contractual Total
Commodities Costs:		
Description		
Materials and supplies for genetic analysis		
		Commodities Total

FY03

Project Number: 03190
 Project Title: Construction of a Linkage Map for the Pink Salmon Genome
 Name: University of Montana

Prepared: July, 2002

FY 03 EXXON VALDEZ TRUSTEE COUNCIL PROJECT BUDGET

October 1, 2002 - September 30, 2003

New Equipment Purchases:		Number of Units	Unit Price	
Description				
Those purchases associated with replacement equipment should be indicated by placement of an R.		New Equipment Total		
Existing Equipment Usage:		Number of Units		
Description				
Hitachi FMBIO 100 Fluorescent Imaging Scanner		1		

FY03

Project Number: 03190
 Project Title: Construction of a Linkage Map for the Pink Salmon Genome
 Name: University of Montana

Prepared: July, 2002