ANNUAL PROJECT REPORT ANNUAL REPORT #: 3

CALFED SCIENCE PROGRAM PROJECT NUMBER: SCI-05-G09-318
PROJECT TERM: SEPTEMBER 26, 2005 – DECEMBER 31, 2008
PROJECT TITLE: 'IS SEX-REVERSAL OF FALL CHINOOK SALMON A SYMPTOM OF GENOTOXICITY?'

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Funding Source: U.S. Geological Survey

Project Location: University of CA, Davis, Genomic Variation Lab

Brief Description of Project:
The focus of our CALFED proposal is to discriminate which chromosomal mechanism is responsible for producing 'apparent' sex-reversed Chinook salmon. We are collaborating with Dr. Ruth Phillips (WA St. Univ., Vancouver) to cytogenetically evaluate progeny produced by normal and 'apparent' XY-female Chinook salmon.

Primary Objective to be Achieved: The primary goal of this research project is to facilitate implementation of the CALFED Ecosystem Restoration goal for fall-run Chinook salmon with respect to the relative importance of chemical stressors on population viability and genetic diversity. We will determine the chromosomal mechanism responsible for producing phenotypic female fall-run Chinook salmon in the Central Valley that test positive for Y-chromosome specific genetic markers. Using established fish-rearing and molecular genetic protocols developed during a previous study we will perform genetic and cytogenetic analyses of offspring produced from genetically normal and 'apparent' XY-female fall-run Chinook salmon. This research project involves three tasks. First, we will harvest gametes and evaluate genotypic sex of phenotypic female and male fall-run Chinook salmon collected from the Merced River Fish Hatchery using two Y-chromosome specific markers, OtY1 and growth hormone pseudogene. By performing genetic screening of sex to determine which of the phenotypic females have a male genotype (XY-females) and which are genetically normal (XX) females, putative gamete donors will be selected for use in controlled breeding experiments. Second, we will evaluate the genetic sex of offspring produced from these crosses using the Y-chromosome markers, plus a recently published Y-chromosome marker, OtY2(WSU). Gross gonad morphology will be observed via necropsy to ascertain the phenotypic sex of offspring. In addition, whole blood will be collected from individuals to be used to create lymphocyte cultures for cytogenetic analyses. Third, the offspring produced between 'apparent' XY-female and normal male fish will be compared cytogenetically with the progeny of normal male and female fish using a previously established cytogenetic method. This will allow us to determine the chromosomal mechanism responsible for producing 'apparent' XY-female fall-run Chinook salmon. Evaluating the chromosomal changes incurred by XY-females should remove uncertainty regarding whether or not XY-female fish negatively impact population genetics and persistence and if they are a symptom of genotoxicity experienced by fall populations due to exposure to environmental contaminants.

We will use a hypothesis driven approach to reduce uncertainty regarding the negative impact that 'apparent' sex-reversed individuals have on populations of this at-risk species. We will address the following hypotheses:

1. Is the 'apparent' sex-reversed phenotype observed in fall-run Chinook salmon due to a Y- to X-chromosome/autosome translocation?
    a. H_o: Male & female offspring of apparent XY-female fall-run Chinook salmon produce patterns of chromosome staining that are consistent with recombination of the male (Y-chromosome) specific markers to the X-chromosome/autosome.

2. Is the 'apparent sex-reversed' phenotype observed in fall-run Chinook salmon due to a Y-chromosome that has had the sex determining region deleted or inactivated?
    a. H_o: Male & female offspring of apparent XY-female fall-run Chinook salmon produce patterns of chromosome staining that are consistent with a Y-chromosome that lacks a functional sex-determining region.

3. Does the OtY2(WSU) Y-chromosome specific marker exhibit the same pattern of inheritance as two other male-specific markers, OtY1 and the growth hormone pseudogene, in a controlled mating between an 'apparent' XY-female and a genetically normal male?
    a. H_o: The Y-chromosome specific marker OtY2(WSU) is inherited in a manner consistent with Mendelian segregation. Specifically, does a controlled cross between a normal male (XY) and an 'apparent' XY-female produce a 1:3, female to male, genotypic sex ratio in the offspring?

The study of genetic mechanisms that alter sexual development of Central Valley Chinook salmon is within the CALFED Program goals of protecting and improving At-Risk Native Species populations and the CALFED Science Program goal of addressing uncertainties that influence management and developing performance measures. Our project will contribute information to the specific priority topic areas of life cycle models and population biology of, and relative stresses on target fish species.

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BUDGET SUMMARY (All tasks should exactly match those identified in the project Scope of Work.)

TASK/SUBTASK	% COMPLETE (BY DOLLARS)	AMOUNT INVOICED (CURRENT FISCAL YEAR)	AMOUNT INVOICED TO DATE (ALL YEARS)	PROJECTED EXPENDITURES (NEXT 6 MONTHS)
Task 1	100	$12,609.38	$42,609.38	
Task 2	100	$27,478.13	$58,478.13	
Task 3	100	$19,678.88	$42,646.88	
Totals	100	$59,766.38	$143,734.38	

PROJECT STATUS TO DATE (BY TASK)

This section should be a cummulative overview of the activities performed to date and include both current and past information for each task. Please list all new information at the top of each task section so that it is clear which information is the most recent. At the end of the project, this section will serve as a full historical record of all activities performed on the project.

TASK 1: REARING OF FAMILIES AND SAMPLE COLLECTION

Dr. Williamson and Dr. May have updated their training regarding experimental animal use and care guidelines and responsibilities by taking a 30 minute on-line course as set forth by the University of California. By doing so our Animal Use and Care Protocol will remain current in accordance with University of California policy and standards.

We have reapplied for and have been granted an extension of our University of California Davis Animal Use and Care Protocol (#9917) in the event that we plan to perform more controlled crosses.

We have successfully performed controlled breeding experiments using gametes from genetically normal and apparent XY-female fall-run Chinook salmon harvested at the Merced River Fish Hatchery during November, 2004. So that genetic sex could be determined, a fin-clip was taken from all adults sampled as potential gamete donors. All potential male (N=3) and female (N=83) parents were genotyped using Y-chromosome specific molecular probes. Out of 83 phenotypic females examined, six had a male genotype (XY-females). All three phenotypic males had a male genotype. A breeding matrix was used to create separate, single parent pair families using eggs from all six of the XY-females, four normal females, and milt from two males. Fertilization and incubation of eggs, and rearing of juvenile fish was carried out at the Center for Aquatic Biodiversity and Aquaculture (UC Davis).

During the rearing period, offspring from two families (one from a normal female, the other from a XY-female) were selected at random, individually marked, fin-clipped for genetic analysis of sex, and housed in separate tanks until they were sampled as pre-smolts in May and July, 2005. Since fin-clipping permitted offspring to be genotyped, their genetic sex was known before each was later sampled for cytogenetic analysis. The first set of offspring (6 offspring from a normal female parent) sent to Dr. Ruth Phillips (WSU) for cytogenetic analysis were sampled in May, 2005. This first group of fish were sent primarily as a 'test run' to determine if sufficient volume of whole blood could be obtained from each individual so that primary lymphocyte cultures could be established. Fortunately, this effort was successful & cytogenetic data were acquired from these and subsequently examined offspring (discussed below).

TASK 2: GENETIC ANALYSIS OF SEX

Several additional microsatellite loci have been identified as belonging to the same linkage group as Omy7INRA in rainbow trout (R. Phillips, WSU, personal communication). Out of the several loci identified, the following can be amplified by PCR in Chinook salmon: Omm1087 (Rexroad et al. 2002a), Omm1164 (Rexroad et al. 2002b), Ogo4UW (Olsen et al. 1998), Omm1305 (Palti et al. 2002), Omm5099 (Rexroad et al. 2003), and Omy3DIAS (Holm et al. 2001). Segregation analysis of these microsatellite loci will be performed for the same families used for cytogenetic analysis, as well as additional families produced by normal and XY-female Chinnok obtained from a previous study by Williamson and May (2005).

Several microsatellite loci have been identified as belonging to the same linkage group as Omy7INRA in rainbow trout (Danzmann et al. 2005). Since Phillips et al. (2005) have recently demonstrated that Omy7INRA is sex-linked in Chinook, the other microsatellites in the same linkage group may be sex-linked as well. Possible linkage differences using a subset of these loci (Omm1077, Omm1318, Omm1220, and Omm1156) were investigated in normal and XY-female families. The loci were chosen on the basis of the ability to be amplified in Chinook salmon. Segragation analysis may provide additional clues regarding whether XY-female Fall Chinook are the result of an intra- or inter-chromosomal rearrangement involving the alleged primary sex determining locus SEX and the microsatellite loci examined. Segregation analysis of microsatellite loci Omy7INRA, Omm1077, Omm1318, Omm1220, and Omm1156 have been completed for the same families used for cytogenetic analysis, as well as additional families produced by normal and XY-female Chinnok obtained from a previous study by Williamson and May (2005).

We are performing segregation analysis for five microsatellite loci (including Omy7INRA) in families produced by normal and ‘apparent’ XY-female Chinook salmon.

Genotypic and phenotypic sex data was evaluated at three genetic sex markers in families of Chinook salmon created in 2003 and 2004. It is possible that this information may be incorporated into a collaboative peer-reviewed manuscript that compares the performance of the three genetic sex markers in several different seasonal runs of Chinook salmon in California Rivers. Since the genetic sex markers are incorporated into large scale studies of Chinook salmon throughout the state, elucidating the degree of error associated with each marker will allow for more accurate estimation of demographic data from genetic information. To date, collaborators on the manuscript include Jason Baumsteiger (University of Idaho, Moscow) and William Ardren (USFWS).

Several sex-linked microsatellites have been discovered in rainbow trout (*Oncorhynchus mykiss*) by Danzmann et al. (2005). Phillips et al. (2005) has recently demonstrated that one of these microsatillites, Omy7INRA (Gharbi and Guyomard, INRA, France, *personal communication*), is sex-linked in Chinook. We are performing segregation analysis for five microsatellite loci (including Omy7INRA) in families produced by normal and ‘apparent’ XY-female Chinook salmon. We plan to test the hypothesis of no difference in the pattern of inheritance of sex-linked microsatellites between the offspring of normal and ‘apparent’ XY-female Fall-Run Chinook salmon. Through use of both FISH and segregation analyses it should be possible to reconcile whether a Y- to X-chromosome/autosome rearrangement (recombination of sex markers or translocation of portion of Y), or a Y chromosome containing an inactivated sex determining region is responsible for producing apparent XY-female Fall-Run Chinook in California Rivers.

Both the genotypic and phenotypic sex data was obtained for offspring sampled for cytogenetic analyses. Genetic sex of offspring was evaluated using three different Y-chromosome specific probes: OtY1, growth hormone pseudogene (GH-psi), and OtY2(WSU). Previous work by other researchers (Brunelli and Thorgaard 2004) indicated that OtY2(WSU) is not in physical proximity to either of the other two Y-chromosome specific probes in Chinook salmon. Accordingly, the third marker was incorporated into our analyses as it can provide valuable insight into the chromosomal mechanism responsible for 'apparent' sex-reversal of Chinook salmon. Since necropsy was performed to ascertain gross gonad morphology for sampled offspring, phenotypic sex could be compared with genetic sex using three different Y-chromosome markers. Comparing offspring genotypic and phenotypic sex permitted cytogenetic analyses to be focused on individual offspring that would be of particular interest. For instance, by targeting phenotypic female offspring that did or did not carry the male specific genetic markers one could then make a qualitative comparison between offspring that either did, or did not carry a chromosomal rearrangement.

The Y-chromosome marker OtY2(WSU) was also used to genotype a subset of normal and XY-female Fall Chinook families created via controlled breeding experiments in 2003. The offspring of these families had been already genotyped at the OtY1 and GH-psi loci as part of an earlier research project (Williamson and May 2005). Furthermore, several of the families also contained additional necropsied, but non-genotyped siblings. By genotyping the previously examined families using OtY2(WSU) and genotyping their remaining siblings at all three markers, sample size for each family was increased, thereby permitting increased power to resolve inheritance pattern differences between all three markers in both normal and XY-female Fall Chinook salmon.

TASK 3: CYTOGENETIC ANALYSIS OF OFFSPRING

Fluorescent In-Situ Hybridization (FISH) has been used to evaluate the patterns of chromosomal staining produced by fluorescent-labeled, Y-chromosome specific probes in a subset of male and female siblings from normal and XY-female Fall Chinook salmon. Multiple chromosome and sex marker rearrangements between the male and female offspring of XY-females have been observed.

More offspring from each of the families will need to be cytogenetically evaluated before preliminary results are presented to the larger scientific and resource management communities. As further analyses to assess the nature of the genetic change responsible for producing XY-females provide more data, the types of molecular genetic techniques required to further resolve the exact kind(s) of genetic changes involved in the sex-reversal phenomenon will be adapted to our program.

PROJECT-WIDE STATUS

ACHIEVED OBJECTIVES, FINDINGS, AND CONTRIBUTIONS:

Achived Objectives

Task 1- The primary activities of Task 1 have been completed. The remainder of percent effort will be devoted to reporting, scientific papers, and winding up this overall project.

We have maintained our current Animal Use and Care Protocol in accordance with University of California policy and standards.

Successful collection of gametes from Fall Chinook salmon, genetic screening of sex of potential parents, performing controlled breeding experiments, rearing of juvenile fish to the pre-smolt stage was achived. Collecting fin-clip and blood samples from offspring used to determine genetic sex and examine fluorescent staining patterns of chromosomes, respectively, was successful.

Task 2- Segregation analysis of microsatellite loci Omm1087, Omm1164, and Omm5099 has been performed on experimental and control families of fall-run Chinook salmon previously examined for sex chromosome segregation and cytogenetic analysis. The primary activities of Task 2 have been completed.

Experimental and control families of fall-run Chinook salmon previously examined for sex chromosome segregation and cytogenetic analysis were successful amplied using additional microsatellite loci (Omm1087, Omm1164, Omm5099) that may be linked to sex in Chinook salmon. We are continuing our analysis of the segregation pattern with respect to sex for the additional loci in each family of fish.

The results of segregation analysis of microsatellite loci Omy7INRA, Omm1077, Omm1318, Omm1220, and Omm1156 have been summarized, combined with the cytogenetic analyses of offspring produced by normal and XY-female Fall Chinook (Task 3), and have been presented in a draft manuscript intended for publication in a peer-reviewed journal.

Primer sequences for rainbow trout sex-linked microsatellite loci were obtained, the primers ordered, and PCR assay conditions developed for each locus in Chinook salmon.

Offspring of controlled crosses performed in 2004 (& sub-sets from 2003) were successfully genotyped using all three Y-chromosome markers. Genotypic sex data from all three markers and phenotypic sex data have been compared between families of normal and XY-female Fall Chinook.

Task 3- The results of our findings have been published in the Journal of Heredity.

Cytogenetic analyses of offspring produced by normal and XY-female Fall Chinook using the fluorescent insitu hybridization probes GH-Ψ, OtY1, and Omy7INRA have been completed. The cytogenetic and segregation analysis results have been presented in a draft manuscript intended for publication in a peer-reviewed journal.

Several offspring from both a normal and XY-female have been examined cytogentically. Preliminary results have been obtained. Further data are being collected.

Findings-

1. For microsatellite loci Omm1087, Omm1064, and Omm5099 neither male, nor female parent linkage was observed in the families produced by normal and XY-female Chinook salmon.

2. The FISH analyses suggest that XY-female Fall Chinook salmon in California are not the product of a Y chromosome to autosome translocation.

3. Fluorescent in situ hybridization (FISH) experiments with the Y chromosome probes OtY1 and GH-Ψ revealed that the apparent XY-female examined transmitted a "Y-like" chromosome with an attenuated OtY1 and GH-Ψ signal to half of its offspring.

4. Inheritance patterns of Omy7INRA were consistent with this locus being closely linked to GH-Ψ in males and in females carrying the "Y-like" chromosome. One of the rainbow trout loci (Omm1077) evaluated revealed close linkage in males, but not XY-females.

5. A different inheritance pattern for Y-chromosome marker OtY2(WSU), compared to that for OtY1 & GH-psi, has been observed in the offspring of XY-female families.

6. One microsatellite, Omy7INRA, appears to exhibit sex-linkage in both males and XY-females in several of the families examined. Two other sex-linked microsatellites, Omm1077 and Omm1318, had similar segregation patterns regardless of which family (normal or XY-female) was evaluated;

   Multiple chromosomal rearrangements have been observed the male and female offspring a XY-female.

7. Another round of breeding experiments that include cytogenetic analysis of adult gamete donors may provide another means to study chromosomal rearrangements in more depth.

Contributions- To date our results do not clearly discriminate between the other alternative explanations for XY-females, namely, recombination of Y-specific markers between the sex chromosomes, or a Y chromosome with a dysfunctional or missing sex determining region. However, the data we have collected suggest that DNA sequencing of the regions adjacent to the repetitive DNA segements that harbor OtY1 in XY-females may lead to the discovery of novel markers close to the sex determining region of the Chinook Y chromosome. This experimental approach will be followed for the separately funded USFWS cooperative agreement #113322J006.

The amendment request to the USFWS cooperative agreement #113322J006 and this CALFED project to examine the chromosomal mechanism responsible for producing "apparent" sex-reversed Chinook salmon have non-overlapping and complementary goals. Because each project uses a different experimental approach to evaluate separate hypotheses related to apparent sex-reversal of Chinook salmon, each project has the potential to guide the other as new information is produced. The information obtained from evaluating gross chromosomal rearrangements will guide concurrent (and separately funded) USFWS project to obtain higher resolution of the genetic changes incurred by XY-females. Specifically, knowledge of gross chromosomal rearrangements will be used direct sequencing efforts to a particular region of the Y-chromosome, or away from others.

PROBLEMS OR DELAYS ENCOUNTERED:

Amplification of heterologous loci in Chinook salmon-

Inheritance testing of microsatellite locus Omm5099 could not be performed in experimental family 181xA (produced by an XY-female) since the male parent used to produce the family was homozygous at Omm5099. Homozygosity in either parent precludes testing of inheritance pattern of alleles in the offspring. However, since multiple families (both control and experiemental) were used for testing inheritance at Omm1087, Omm1164, and Omm5099, allelic inheritance could still be tested for Omm5099.

Six additional microsatellite loci were evaluated as potential additional markers to analyze inheritance in experiemental families of fish. We have continued to develop PCR assay amplification conditions for these loci. We tested the ability to amplify microsatellite loci, Omm1087, Omm1164, Ogo4UW, Omm1305, Omm5099, and Omy3DIAS in fall-run Chinook salmon. Those loci (Omm1087, Omm1164, Omm5099) that were successfully amplified and exhibited polymorphism were used to test inheritance in the same families that had been examined previously.

Amplicication of DNA using PCR primers developed in other closely related species is not necessarily straightforward. While the loci in question may be conserved evolutionarly (inherited from a common ancestor in the distant past) the locus may not be polymorphic in the species being studied (rendering it non-informative with respect to the analysis of inheritance within a family). In addition, the conditions under which PCR amplification (hence detection) of a microsatellite locus using DNA primers designed from the same locus in a closely related species (heterologous locus) is successful need to be developed on a locus by locus basis.

Blood drawing from juvenile fall chinook salmon- In order to obtain a sufficient volume of whole blood from juvenile fish so that a lymphocyte culture could be established, required using a different sampling technique than the one given in our original proposal. Caudal vein puncture was not particularly well suited for sampling blood from juvenile fish because of their small size, relatively low blood volume, and extremely short blood coagulation time (~2-3 sec.). This problem was circumvented by rearing fish for another month so that they grew larger, adding a Sodium Heparin (anticoagulant) solution to synringes prior to sampling blood, and using an alternate blood drawing site (cardiac /dorsal sinus).

DELIVERABLES:

6th Bi-Annual Status: A summary of our cytogenetic and inheritance analyses was presented at the 2008 CALFED Bay-Delta Authority Science Symposium, Sacramento, CA, Oct. 22-24, 2008. Title: Characterization of a chromosomal rearrangement responsible for producing ‘apparent’ XY-female Chinook salmon.

Our manuscript regarding the cytogenetic analysis of chromosome rearrangement in California fall-run Chinook may be referenced as follows:

Williamson, K.S., R. Phillips, and B. May. 2008. Characterization of a chromosomal rearrangement responsible for producing ‘apparent’ XY-female fall Chinook salmon in California. Journal of Heredity 99: 483-490.

A pdf file of our manuscript published in the Journal of Heredity will accompany the third annual report sent to the CALFED Technican Contact (Michelle Shouse).

5th Bi-Annual Status: Our maunscript regarding the cytogenetic analysis of chromosome rearrangement in California fall-run Chinook salmon was accepted for publication in the Journal of Heredity. Until the manuscript is published in hard copy it may be referenced as follows:

Williamson, K.S., R.Phillips, and B. May. 2007. Characterization of a chromosomal rearrangement responsible for producing ‘apparent’ XY-female fall Chinook salmon in California. Journal of Heredity. (*in press*).

Once the manuscript has been published, we will provide an electronic copy along with our third annual report.

3rd & 4th Bi-Annual Status: A draft manuscript regarding our research results is currently under internal review at the NMFS/NOAA Fisheries NW Fisheries Science Center. Once the internal review and editing is complete, we intend to submit the manuscript for peer-review and eventual publication in the Journal of Heredity. This journal was chosen because it has broad readership in the genetics community at large as well as within the fisheries genetic community.
Title: Characterization of a chromosomal rearrangement responsible for producing apparent XY-female fall Chinook salmon in California

2nd Bi-Annual Status: Cytogenetic analyses have been presented at regional and national scientific conferences under the title of "Cytogenetic analysis of 'apparent' XY-female Fall Chinook in California":

a) 2006 CALFED Bay-Delta Authority Science Symposium, Sacramento, CA, Oct. 25, 2006;
b) 136th Annual American Fisheries Society Meeting, Lake Placid, N.Y., Sept. 13, 2006;
c) 2006 Coastwide Salmonid Genetics Meeting, Santa Cruz, CA, June 23, 2006.

Presentation of the research results to the salmonid genetics and fisheries management communities has fostered considerable dialogue with other researchers and managers.

Furthermore, a manuscript of our research results is in preparation while we are gathering further data regarding the segregation analysis of sex-linked microsatellite loci in Fall Chinook salmon families that were created for this project and from an earlier CALFED funded project.

1st Bi-Annual Status: Semi-annual and annual reports of project status and results; presentation of research results to scientific and natural resource management communities at regional and national conferences

PERSONNEL CHANGES:
NONE.

CONTRACT MODIFICATIONS:
NONE.

NOTES/OTHER:

Collaboration with parallel CALFED project
Dr. Williamson presented his research results to Drs. Loyo-Rosales and Sedlak and their collaborators at the University of California Berkeley in May 2007.

Dr. Jorge Loyo-Rosales and Dr. Kevin Williamson have discussed presenting a 'joint' poster at the 2007 CALFED Bay-Delta Authotity Science Symposium. The poster is intended to summarize both the future directions and current results of our parallel research projects. In addition, Dr. Williamson has been invited to attend a meeting with Dr. Loyo-Rosales and his collaborators at the University of California Berkeley in May 2007.

Dr. Bernie May and Dr. Davis Sedlak met at the University of California at Davis.

Met with Dr. Kolodziej to discuss researech results of our projects, and meet the post-doctoral researcher taking over his project. I have mailed to Dr. Kolodziej a DVD recording of a talk given by Dr. Michael Skinner (Washington State University) to staff and scientistsof the Northwest Fisheries Science Center in November 2006. The information given in Dr. Skinner's talk about transgenerational genetic effects of a pesticide on mice may provide Dr. Kolodziej with additional future research ideas for his ecotoxicology related work with fish species in California Rivers.

The tasks for this CALFED funded project are being conducted in collaboration with those of Dr. David Sedlak, the principal investigator for the research proposal, "Identifying the Causes of Feminization of Chinook Salmon in the Sacramento and San Joaquin River System", which has also been approved for funding by CALFED. Dr. Williamson has also been in contact with Drs. Sedlak and Kolodziej to discuss the details of earlier as well as current research results regarding apparent sex-reversal of Central Valley Fall Chinook.

References:

Brunelli, J.P., G.H. Thorgaard. 2004. A New Y-Chromosome Specific Marker for Pacific salmon. Transactions of the American Fisheries Society 133: 1247-1253.

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