

II. Plan of work (by task)

Task 1 - Rearing of families and sample collection

We have successfully performed controlled breeding experiments using genetically normal and apparent XY-female fall-run Chinook salmon during the last three spawning seasons (2002 to 2004). Once additional families have been reared to the pre-smolt stage of development we will have sufficient material with which to test hypotheses. The control and experimental families of fish produced from genetically normal females and XY-females, respectively, will be reared at the Center for Aquatic Biodiversity and Aquaculture (UC Davis).

Sample collection for artificial crosses— Fin-clips for genetic analysis and gametes from fall-run Chinook salmon returning to the Merced River Fish Hatchery (MRH) will be collected with the assistance of California Department of Fish and Game personnel during the spawning period (Late October to Early December) of these fish. . Approximately 2 cm² of caudal fin tissue near the caudal peduncle will be excised with scissors from each fish sampled and placed into separate, labeled coin envelopes. Eggs from phenotypic females will be expressed into pre-labeled plastic urine analysis cups, sealed and immediately placed on a raised platform within an ice chest. Milt from three phenotypic males will be expressed into labeled Zip-Loc® bags and similarly stored. Tissue samples and gametes will be stored between 5-8°C while transported back to the University of California Davis Genomic Variation Lab (GVL) for genetic analysis and use in controlled breeding experiments conducted at the Center for Aquatic Biodiversity and Aquaculture (CABA), respectively.

Breeding experiments - The eggs from each single phenotypic female fish selected will be split into roughly two equal portions and placed into separate styro-foam containers. Aliquots of eggs will be separately fertilized with milt from separate, single, genetically normal males. In this manner, each family of fish will have only two parents. Hatchlings from individual families will be incubated at 12°C for approximately 45 days (just before swim up stage) in Heath trays and will be transferred to separate, larger rearing tanks.

Collection of genetic samples from offspring - Once development has proceeded to the pre-smolt stage, whole blood will be drawn via caudal vein puncture into sterile, heparinized collection tubes from euthanized individuals using standard procedures (Phillips 2005). Whole blood from separate individuals will be shipped overnight to Dr. Ruth Phillips (participating investigator) for cytogenetic analysis. During blood collection for each individual a tissue sample will be taken for analysis of genetic sex, and a necropsy will be performed to ascertain gross gonad morphology.

Task 2 - Genetic analysis of sex

Genetic screening to detect apparent sex-reversed male (XY female) fish – The selection criterion for sets of gametes to be used in artificial crosses will be based on the sexual genotype at the Growth Hormone pseudogene and OtY1 loci of putative parents. All phenotypic female and male fall-run Chinook sampled will be genetically screened by polymerase chain reaction (PCR) assays using the OtY1 primers developed from Chinook salmon by Devlin et al. (1994) and the Growth Hormone pseudogene (GH-Ø) primers developed by Du et al. (1993). DNA fragments amplified by PCR were resolved on a 5.5% acrylamide-7M Urea gel and imaged by a MJ Research BaseStation (MJ Research, San Francisco, California). Individual genotypes will be scored using Cartographer® software as well as manually verified for every individual genotyped. Chinook that test positive for a 209 base pairs (bp) PCR fragment and that did not produce a series of larger PCR products characteristic of the OtY1 locus in females (Devlin et al. 1994) and that tested positive for a 276 bp band indicative of the GH Ø (Du et al. 1993) were scored as

being positive for having the Y-chromosome markers (genetic males). In the case where a fish that had ovaries produced both a robust 209 bp PCR fragment (OtY1) and the 276 bp fragment (GH-Ø), that fish was scored as a XY-female. When the larger PCR fragments characteristic of females were present and the 209 bp PCR fragment was not present and the 276 bp band indicative of the GH pseudogene was also absent, the fish was scored as being negative for having the Y-chromosome markers (genetic female).

Genetic sex of each individual will be evaluated (as above) using the Y-chromosome specific probes OtY1 and growth hormone pseudogene as well as another Y-chromosome marker OtY2(WSU) recently developed by Brunelli and Thorgaard (2004). Since there is evidence that OtY2(WSU) is not in physical proximity to either of the other two Y-chromosome specific probes in Chinook salmon (Brunelli and Thorgaard 2004) we have decided to incorporate this new marker for genetic sex into our analyses. It is possible that the pattern of inheritance of the OtY2(WSU) locus differs from the other two in families produced by 'apparent' XY-female fall Chinook salmon. Using the additional marker may provide valuable insight into the chromosomal mechanism responsible for 'apparent' sex-reversal of Chinook salmon.

Task 3 - Cytogenetic analysis of offspring

Metaphase chromosome preparations will be made from lymphocyte cultures using standard procedures (Phillips 2005). The Y-chromosome will be identified using either a plasmid clone containing OtY8 cosmid and or a cosmid clone containing the GH-Y gene from Chinook salmon (provided by Robert Devlin, Fisheries and Oceans Canada) or both. High molecular weight DNA will be extracted from the plasmid using standard procedures and from the cosmid using a Qiagen kit. Fluorescence in situ hybridization (FISH) analysis will be done as described in Stein et al., (2001) with minor modifications. The clones will be labeled either with Spectrum Orange (Vysis, Inc.) or digoxigenin (Roche, Inc.). Blocking repetitive sequence (Cot1 DNA) is added to the probe and the probe and chromosomal DNA are thermally denatured. The denatured probe is added to the slide that is hybridized overnight under controlled stringency. After a series of post-hybridization washes, slides are counterstained with DAPI and viewed under fluorescent illumination. For digoxigenin labeled probes, antibodies to digoxigenin (Roche, Inc.) diluted in phosphate buffered saline (PBS) are added to the slides after the post-hybridization washes and the slides are incubated for 45 minutes before a final series of washes and application of the DAPI counter stain. Fluorescent images are captured separately for each fluorochrome with a digital camera and combined using the CYTOVISION image analysis program (Applied Imaging, Inc).