May 11, 2010

To Whom It May Concern,

We are submitting a thoroughly revamped narrative for project 2009-005-00 “Influence of Environment and Landscape on Salmonid Genetics”. The narrative has been extensively revised to address comments provided by the ISRP and NPCC. The primary objectives of the study have not changed, but supporting details and study design have been improved.

The revision process included multiple drafts and reviews by internal CRITFC staff, external scientists (Chuck Peven and Ewann Berntson), and our BPA COTR (Barbara Shields). This effort was intended to get comments from outside experts and partners in the Columbia River Basin. All parties involved believe that this revised narrative represents a substantial improvement and that this study will be a useful contribution to fisheries science and management.

Specifically, our revised narrative focused on four main areas brought up by ISRP and NPCC:
1) more background information for the two initial traits of interest (thermal tolerance and smoltification)
2) more details regarding our approach to identifying candidate markers in Objective 1
3) improved study design for Objective 2 to validate candidate markers
4) information regarding management implications of this study
We decided to integrate these revisions throughout the entire narrative in order to create a single cohesive document rather than a separate study plan that was not clearly integrated into the narrative.

I appreciate your support of this research, and I anticipate significant contributions from this study to fisheries science and management over the course of the project.

Shawn Narum, PhD
Lead Geneticist
Columbia River Inter-Tribal Fish Commission

Narrative

Influence of Environment and Landscape on Salmonid Genetics

Table 1. Proposal Metadata

Project Number	2009-005-00
Proposer	Columbia River Inter-Tribal Fish Commission
Short Description	Influence of Environment and Landscape on Salmonid Genetics
Province(s)	Basinwide
Subbasin(s)	Basinwide
Contact Name	Shawn Narum
Contact email	nars@critfc.org

Information transfer:

A. Abstract

Environmental and landscape features can greatly contribute to population structure, life history diversification, and adaptation of salmonids. This proposal combines two projects from the Fish & Wildlife Program Accords with the following objectives: 1) Environment & Landscape Genetics – Evaluate genetic structure of natural populations of salmonids relative to their environment and identify candidate markers associated with traits that are related to adaptation of steelhead and Chinook salmon populations (i.e., smoltification and thermal tolerance); and 2) Controlled Experiments – laboratory/hatchery experiments with controlled environmental variables to validate phenotypic response of fish with given genotypes. This proposal consists of two parts, the first involving both Chinook salmon and steelhead, the second only steelhead. The first part will be conducted in stages: first, mining extant genetic data; second, genotyping new genetic markers (SNPS); third, screening archived tissue samples with these new markers; then, analyzing all these markers for concordance or discordance with environmental and geological characters across the landscape. From this step, we will then identify SNPs (and thereby loci) that are not conforming to neutral expectations, but in concordance with gradients of one or more environmental variables. These putative or potential 'candidate markers' under local selection pressure will be further explored in Part 2 as a validation step under controlled circumstances.

This information will facilitate understanding of adaptation of natural populations of salmonids to their environment. We expect this to benefit future management/recovery of natural and supplemented populations, along with reintroduction programs. This study will provide a basis for monitoring biologically relevant genetic diversity in salmonid populations in contrast to selectively neutral levels of genetic diversity that are typically evaluated. This information could be highly useful for hatchery programs that are uncertain of which broodstock may offer the best chance at recovery for anadromous steelhead or those that are adapted to local environmental conditions and pathogens.

The work related to both objectives will be completed by CRITFC and University of Idaho (UI) staff at the Hagerman Fish Culture Experiment Station. Geneticists with CRITFC have adequate expertise to complete these objectives, and have published related peer-reviewed papers (Narum et al. 2008; Campbell et al. 2008; Narum and Campbell 2009).

B. Technical and/or scientific background

Objective 1) Environment & Landscape Genetics of Natural Populations

Environmental and landscape features can greatly contribute to the population structure, life history diversification, and local adaptation of organisms in aquatic habitats (reviewed in Storfer et al. 2006). Geographic barriers to dispersal include recent events that may have been human induced (e.g., dams) as well as ancient events such as glaciations and formation of mountain chains (e.g., Castric et al. 2001). However, other environmental characteristics such as elevation, temperature, forest cover, and precipitation may influence distribution, adaptation, and gene flow of species (Dionne et al. 2008; Narum et al. 2008). For example, the geographic distributions of species ranges' are often determined by thermal tolerance (Brannon et al. 2004) and may necessitate adaptations for survival in extreme environments (Rodnick et al. 2004).

In this study, we plan to screen a suite of approximately 100 SNP markers (e.g., Campbell et al. 2009) in natural populations of steelhead and Chinook salmon for which we have previous information regarding traits of interest (i.e., thermal tolerance and smoltification). Markers within the suite will be split into categories of putative candidate or neutral loci for analyses of local adaptation and gene flow, respectively.

Screening with many genetic markers provides the opportunity to investigate local adaptation in natural populations and identify candidate genes under selection (Beaumont and Nichols 1996; Beaumont and Balding 2004; Excoffier et al. 2009). This has become a commonly employed approach in ecological and population genetics studies to detect outlier loci that are putatively under selection (e.g., Vasemagi and Primmer 2005; Nosil et al. 2008). Additionally, correlation methods can be highly informative to identify markers in coding and cis-regulatory regions of known functional genes that are associated with specific selective pressures or phenotypes (Lyman and Mackay 1998; Chase et al. 2009; Torgerson et al. 2009). With increasing genomic information available for non-model organisms, single nucleotide polymorphisms (SNPs) have begun to see increased use as genetic markers for population genetic studies (e.g., Morin et al. 2004). These sequence polymorphisms are densely scattered throughout the genome of most organisms, and are commonly observed in both coding and non-coding regions of functional genes making them ideal markers to study adaptive molecular variation (e.g., Akey et al. 2002). In a large suite of unlinked SNPs that are distributed across the genome (e.g., Campbell et al. 2009), it is possible to utilize both functionally neutral and adaptive markers within a single study. This combination of information provides a powerful approach to study questions in ecological genetics since both demographic processes (i.e., gene flow and genetic drift) and local adaptation (i.e., selection) may be inferred.

While candidate markers under selection can be used to address local adaptation in natural populations, the inclusion of neutral markers also provides the opportunity to evaluate gene flow among populations in relationship to geological or environmental barriers. The study will also provide resources for evaluating the maintenance of biologically relevant genetic diversity in hatcheries. A variety of statistical models have been developed to address specific questions related to genetic structure due to environment and landscape features (reviewed in Manel et al. 2003; Storfer et al. 2006). For example, ordination models with canonical

correspondence analysis have been used as an alternative to Mantel tests to simultaneously evaluate drainage, altitude, and human impacts to genetic diversity of salmonid fishes (Angers et al. 1999; Costello et al. 2003). Since many environmental features are inter-correlated, multivariate modeling of parameters related to genetic structure can be employed with tools such as GESTE (Foll and Gaggiotti 2006). Recent applications of interpolation models that utilize multivariate analyses such as principal components analysis (PCA) have also demonstrated that habitat and landscape features can identify and predict spatial patterns associated with restricted gene flow (Piertney et al. 1998). When PCA results are interpolated and overlaid with GIS data, synthesis maps can identify genetic patterns related to landscape (e.g., Narum et al. 2008). In this study, we plan to apply these approaches to better understand environmental genetics of steelhead and Chinook salmon in the Columbia River.

Objective 2) Controlled Experiments in Wet Laboratory/Hatchery Setting

Controlled experiments will be used to confirm and expand results obtained from the first objective of this study. Laboratory or hatchery settings allow for controlling environmental variables and observation of phenotypic response in individual fish. Thus we will be able to evaluate directly the phenotypic response of environmentally influenced traits in fish with a specific genotype under a controlled environment. This approach will help to validate candidate markers identified in objective 1 and determine the potential to model local adaptation of natural populations based on allele frequencies at candidate markers. This validation step eliminates the variable environment that fish experience in the wild, so observed differences are due to genetic (ultimate) rather than environmental (proximate) causes. The initial phase of this study will evaluate smoltification and thermal tolerance, and disease resistance will be added in future years of the project. This step is necessary to determine the association of specific alleles with patterns of gene expression and/or performance. This allows inferences to be made about specific populations with certain frequencies of the alleles under study.

Smoltification is an important trait to study since resident rainbow trout and anadromous steelhead life history types (*O. mykiss*) may give rise to one another (e.g., Zimmerman and Reeves 2000) and thus there is potential for rainbow trout to contribute to the recovery of ESA listed steelhead stocks in the Columbia River Basin. Current recovery plans do not typically include resident rainbow trout, but this life history type may have the potential to contribute to the larger population. Genetic studies have indicated that resident and anadromous life history types commonly interbreed when found in sympatry (e.g., Docker and Heath 2003; Olsen et al. 2005) but these ecotypes may be genetically distinct in geographically proximate locations with differing environments (Narum et al. 2004; Narum et al. 2008). Both environmental and genetic factors determine if individual *O. mykiss* remain as resident rainbow trout, or undergo the necessary physiological changes (smoltification) to prepare for anadromy (Shapovalov and Taft 1954; Ricker 1972; Randall et al. 1987; Peven et al. 1994). While some of the environmental factors that contribute to smoltification such as photoperiod and temperature have been evaluated (Hoar 1976), the genetic mechanisms that contribute to migratory selection are not well known. Recent studies have confirmed that genetic factors do play a role in smoltification (Thrower et al. 2004; Nichols et al. 2008), and quantitative trait loci (QTL) have been identified that are associated with phenotypic traits of smolts such as silvery appearance and body shape (Nichols et al. 2008). However, further research is needed to extend these studies in order to apply this information to natural populations of *O. mykiss* and recovery of ESA listed populations of steelhead. In this proposal, we plan to identify candidate markers associated with smoltification, validate those markers in controlled environments, and then evaluate allele frequencies of these

markers across populations in the Columbia River Basin. Since these same markers are currently utilized for SNP baselines (Genetic Assessment project 2008-907-00), we have the capability to model results throughout the Columbia River Basin. Ultimately, this research will identify alleles associated with expression of the smolting phenotype and enable us to estimate the relative contribution of resident and anadromous *O. mykiss* to population viability, and identify landlocked populations that retain alleles that make them good candidates for anadromous restoration (e.g. Thrower et al. 2004). Additionally, resident rainbow trout that retain smoltification genes could be used in hatchery programs when anadromous fish are limited, or to reduce inbreeding effects.

Other important traits are the behavioral and physiological responses of fish to environmental stressors such as high water temperature. In recent history, water temperatures in parts of the Columbia River basin have increased due to a wide range of factors including habitat destruction, dams, water diversion, and possibly climate change (Crozier et al. 2008). The physiological response and adaptation of steelhead to these circumstances may be critical to persistence of stocks in the Columbia River Basin. Several studies have identified QTLs for upper thermal tolerance in rainbow trout (Jackson et al. 1998; Danzmann et al. 1999; Perry et al. 2001) clearly demonstrating there is a genetic basis for thermal tolerance. Further, differential gene expression of heat shock protein genes in response to high temperatures have indicated regulatory control under thermal stress (Feder and Hoffman 1999; Heredia-Middleton et al. 2007). In our approach, we will screen reference populations with differing thermal profiles with a panel of 96 SNP markers. Candidate markers from the panel will then be tested in controlled experiments to determine phenotypic response of fish under heat stress as an attempt to validate their role in thermal adaptation. This information will assist our understanding of the ability of populations to adapt to increasing temperatures throughout the Columbia River Basin.

Results from controlled experiments will allow us to validate candidate markers related to multiple traits of interest, and provide confidence that these markers can be used more broadly throughout the Columbia River Basin to model local adaptation. However, it will be necessary to confirm the utility of candidate markers in populations with procedures such as correlation with regional environmental variables (e.g., stream temperature). While the initial years of this study will focus on smoltification and thermal tolerance, future years will incorporate disease resistance/immune response into the project. Additional traits such as growth and run timing may be added over the course of the study.

C. Rationale and significance to regional programs

This research proposal affects all stocks in the Columbia River Basin and therefore is considered a basinwide application. The projects in this proposal address needs for management of natural and supplemented populations, along with reintroduction programs. These needs have been identified in multiple "Reasonable and Prudent Alternatives" (RPA) in the BiOp:

-page 57, RPA No. 41, Preserve genetic resources – This study will provide a basis for monitoring biologically relevant genetic diversity in salmonid populations in contrast to selectively neutral levels of genetic diversity that are typically evaluated.

-page 69, RPA No. 50, Fish population status monitoring – Once candidate markers are identified that correspond to specific traits, we plan to monitor populations throughout the Columbia River Basin to evaluate potential for thermal adaptation and smoltification.

-page 89, RPA No. 63, Monitor hatchery effectiveness – The study will provide resources for evaluating the maintenance of biologically relevant genetic diversity in hatcheries.

-page 89, RPA no. 64, Investigate hatchery critical uncertainties – Candidate markers can be highly useful for hatchery programs that are uncertain of which broodstock fish may

offer the best chance at recovery for anadromous steelhead or those that are adapted to local environmental conditions and pathogens.

D. Relationships to other projects

This research project is directly related to Genetic Assessment of Columbia River Stocks (2008-511-00) since baseline genetic data from that study will be utilized for landscape genetic analyses. Additionally, our project is related to Management Scenarios for Climate Change (2008-514-00) since our study addresses the ability of populations to adapt to increasing temperatures.

Table 2. Relationship to existing projects

Funding Source	Project #	Project Title	Relationship (brief)
BPA	2008-907-00	Genetic Assessment	Baseline genetic data will be utilized in landscape genetics analyses.
BPA	2008-514-00	Management Scenarios for Climate Change	Understanding of adaptation to increasing temperatures.

E. Project history (for ongoing projects)

This is a newly funded project in 2009, with 6 months of prior funding. Preliminary efforts are underway to screen SNP markers in natural populations to identify candidate markers associated with thermal adaptation and smoltification.

F. Proposal biological/physical objectives, work elements, methods, and metrics

Related to both Objectives (1 & 2)
Work Elements:
119. Manage and Administer Projects: Project Management
This will include project administration and contract development.

132. Produce (Annual) Progress Report: Submit Progress Report
The progress report will summarize the project objectives, hypotheses, completed and uncompleted deliverables, problems encountered, lessons learned, and long-term planning.

183. Produce Journal Articles: Submit for Publication
As deemed appropriate by project leaders, results from studies will be submitted for publication in peer-reviewed journals.

185. Produce Pisces Status Reports: Periodic Status Reports for BPA
CRITFC shall report on the status of milestones and deliverables in Pisces. Reports shall be completed either monthly or quarterly as determined by the BPA COTR. Additionally, when indicating a deliverable milestone as COMPLETE, CRITFC shall provide metrics and the final location (latitude and longitude) prior to submitting the report to the BPA COTR.

Objective 1) Environment & Landscape Genetics - Evaluate gene flow of natural populations of salmonids relative to their environment and identify candidate markers associated with traits that are correlated to adaptation of steelhead populations (i.e., smoltification and thermal tolerance).

Work Elements:

156. Develop RM&E Methods and Designs: Determine Landscape and Genetic Variables

Landscape genetics analyses methods will be utilized to evaluate hypotheses that landscape features influence genetic structure and life history variation of Chinook salmon and steelhead. Physical variables such as elevation, stream gradient, water temperature (natal habitat and migration corridors), and geographic distance (from ocean as RKM) of each sample site will be included as potential landscape features that influence genetic diversity. Physical and genetic data will be analyzed following Narum et al. (2008) and other pertinent studies (e.g., Dionne et al. 2008).

157. Collect/Generate/Validate Field and Lab Data: Gather Landscape and Genetic Data

Genetic Data:

This objective does not include collecting new samples, but rather accessing archived tissues and genotypes. Thus, microsatellite and SNP genotypes for Chinook salmon and steelhead will be utilized from existing and ongoing genetic baseline projects for each species. Populations will be analyzed from throughout the Columbia River Basin, depending on availability of tissue samples and information from genetic databases.

Landscape Data:

Landscape features for genotyped populations will be determined using Geographic Information Systems (GIS) analysis of each sample site. This stream layer will provide elevation, migration distance, geographical stream distance among sites, and stream gradient which are all factors that may play a role in the life history of salmonids. Precipitation and temperature for each site will be estimated with simulations from PRISM (Parameter-elevation Regressions on Independent Slopes Model; http://www.ocs.orst.edu/prism/) of the Oregon Climate Service.

162. Analyze/Interpret Data: Landscape Genetics Analyses/Correlation

Tests for correlation among landscape and genetic variables will be completed with Mantel tests and multivariate analyses (e.g., GESTE; Foll and Gaggiotti 2006). Results from principle components analysis will be interpolated across the Columbia River drainage.

161. Disseminate Raw/Summary Data and Results: Distribute Results

Results from landscape genetics analyses will be made available in annual reports and publications. Studies will also be presented at professional meetings and seminars as appropriate.

Objective 2) Controlled Experiments – laboratory/hatchery experiments with controlled variables to validate phenotypic response of fish with given genotypes.

156. Develop RM&E Methods and Designs: Sampling Plan for Gene Expression & Candidate Markers

The first year of this study will begin to address two key traits related to recovery of steelhead, smoltification and thermal tolerance. Expression of genes related to these functions will be quantified in fish under controlled environments, and candidate loci for smoltification and heat thermal tolerance will be genotyped. Subsequent years of this project may include additional

traits such as immune response and growth. The methods section contains further information regarding strains of *O. mykiss* that will be used for experiments in controlled environments.

157. Collect/Generate/Validate Field and Lab Data: Tissue Sampling

Tissues will be sampled at multiple developmental stages from fish reared under controlled environments (temperature and photoperiod). Samples will be collected at 3-4 time periods of development, with up to 30 fish per time (e.g., 3 replicate tanks of 10 fish). Fish will be sacrificed with an overdose of MS-222, and then immediately dissected to remove tissues such as brain, liver, gill, and fin tissues for analysis. Tissues will be stored in RNALater and frozen for RNA extraction. Archived tissues collected from fish in natural populations with varying temperature (cool headwater tributaries vs. warmer mainstem sites) and resident vs. anadromous populations will be identified for genotyping with DNA markers (SNPs).

157. Collect/Generate/Validate Field & Lab Data: Quantify Gene Expression and Candidate Markers

RNA expression of candidate genes identified in objective 1, along with known genes related to these traits (i.e., HSP70 and Na/K-ATPase genes) will be quantified with real-time PCR methods in tissues such as brain, liver, and gill that are sampled from fish that have been raised in controlled environments. Candidate SNP markers will also be genotyped in fin tissues collected from each fish. Approximately 1,500 samples will be analyzed by gene expression and genotyped with SNP markers.

162. Analyze/Interpret Data: Gene Expression Related to Traits

The expression of Na/K-ATPase genes is expected to change relative to photoperiod during smoltification, and we will test for significant differential expression of this and other candidate genes over the course of fish development. As possible, temperature and lunar period will also be evaluated in experiments. Individual genotypes and allele frequencies of candidate SNP markers will also be tested for association with observed phenotypes and gene expression patterns. Candidate SNP markers that are validated with genotype by phenotype observations in controlled studies will then be genotyped across the Columbia River Basin to determine potential of resident fish to contribute to anadromy in natural populations (i.e., predict phenotype based on genotype).

Gene expression of HSP70 is expected to be upregulated in response to thermal stress to act as a chaperone and prevent degradation of proteins. We will test for differential expression of HSP70 and other candidate genes under variable temperature conditions, and their association with survival. Individual genotypes and allele frequencies of candidate SNP markers will also be tested for association with observed phenotypes and gene expression patterns. This information will provide insight regarding genetic variability and the potential of fish to adapt to increasing water temperatures.

161. Disseminate Raw/Summary Data and Results: Distribute Results

Results from gene expression and candidate SNPs will be made available in annual reports and publications. Studies will also be presented at professional meetings and seminars as appropriate.

G. Research – Methods

Objective 1) Environment & Landscape Genetics

Objectives:
- Phylogenetic structure of salmonid populations (based on selectionally neutral genes) will be tested for concordance with landscape features from collection sites (e.g., physical or environmental barriers) that influence vicariance, dispersal and connectivity.
- Candidate markers that vary significantly along gradients of local environmental variables will be investigated for validation as loci under selection, associated with local adaptation (e.g., thermal tolerance, run timing, disease resistance, etc.) of salmonids to differing environmental selection regimes.

Experimental Design
In this study, we plan to screen a suite of approximately 100 SNP markers in natural populations of steelhead and Chinook salmon for which we have previous information regarding traits of interest (i.e., thermal tolerance, Cassinelli and Moffitt 2010; and smoltification, Narum et al. 2008). In a large suite of unlinked SNPs that are distributed across the genome, it is possible to utilize both functionally neutral and adaptive (candidate) markers across populations within a single study. Markers within the suite of 100 SNPs will be split into categories of putative candidate or neutral loci for analyses of local adaptation and gene flow, respectively. While candidate markers under selection can be used to address adaptation to local environmental conditions, the inclusion of neutral markers also provides the opportunity to evaluate gene flow among populations in relationship to geological features influencing dispersal and vicariance. This combination of information provides a powerful approach to study questions in ecological genetics since both demographic processes (i.e., gene flow and genetic drift) and local adaptation (i.e., selection) may be inferred from studying these two classes of loci.

Suites of SNP markers will be genotyped in reference collections of *O. mykiss* and *O. tshawytscha* with known information regarding the traits of interest (potential for anadromy and thermal tolerance). To evaluate association of markers with smoltification, we will utilize samples from the Klickitat River characterized as either primarily resident or anadromous in previous studies (Narum et al. 2008a). As possible, future years of the study will include samples from the Yakima River and John Day River where modeling studies are underway to evaluate smoltification potential (e.g, Courter et al. unpublished data – Cramer Fish Sciences). Similarly, to evaluate thermal tolerance we will use reference collections of *O. mykiss* samples from desert vs. mountain locations from the Snake River (Cassinelli and Moffitt 2010) with corresponding temperature data (desert = Owyhee & Bruneau rivers; mountain = Boise & Payette rivers). Initially, between 10-20 populations from the Klickitat River will be screened related to smoltification, and between 10-16 populations from the Snake River will be tested for association with thermal tolerance. Chinook salmon from thermally adapted populations will also be evaluated in reference to others in the Columbia River. Allele frequencies of SNP markers will be determined from DNA isolated from samples representing multiple age classes from each of the populations of interest. As SNP

baselines expand through the Genetic Assessment project (2008-907-00), this information will be used in further analyses.

Quality Control

Known information from previous studies will be used to validate results determined from this new analysis. This includes testing samples from populations that have been included in previous studies (i.e., Narum et al. 2008; Cassinelli and Moffitt 2010) to determine anadromy or thermal tolerance.

Statistical Analyses

Descriptive Statistics:

Deviation from Hardy-Weinberg equilibrium (HWE) will be evaluated at each locus and population with the Markov Chain Monte Carlo (MCMC) approximation of Fisher's exact test implemented in GENEPOP v. 3.3 (1000 batches with 1000 iterations; Raymond & Rousset 1995). Tests for linkage disequilibrium (LD) between all pairs of loci will also be performed using the MCMC approximation of the exact test in GENEPOP to evaluate if multiple SNPs represent the same gene region. Because multiple comparisons will be involved, correction against Type I error will be made in both tests with the B-Y FDR method (Benjamini &Yekutieli 2001; False Discovery Rate) that provides increased power relative to the Bonferroni method (Narum 2006). Minor allele frequency (MAF), unbiased heterozygosity (H_E), and global F_{ST} will be estimated for each SNP in each collection with GENEPOP.

Outlier loci and deviation from neutral expectations

Since genetic variation at markers linked to genes or expressed sequence tags (ESTs) may be affected by natural selection and thus deviate from neutral expectations, it is possible to identify loci under selection by outlier patterns (i.e., Luikart et al. 2003). Selection that is divergent is expected to result in larger genetic distance among collections than expected relative to neutral markers, and conversely, balancing selection will result in lower genetic distance than expected. Further, phylogenetics of neutral loci will have concordance with phylogeography, but discordance with loci under local selection, which may show concordance with environmental (selection) variables. We will investigate patterns of deviation from neutral expectations among the SNPs with outlier approaches. A rigorous statistical approach will be implemented in LOSITAN (Antao et al. 2008) that simulates a distribution of Fst values under neutral expectations to identify candidates for positive and balancing selection from a plot of average locus heterozygosity versus F_{ST} (Beaumont and Nichols 1996; Beaumont and Balding 2004). Simulations will be run to independently generate a distribution of F_{ST}, based on 50,000 replicates, for all SNPs under an infinite alleles model. The simulation results will then be plotted to represent the median, and the 95% and 99% quantiles. Loci lying outside these quantiles will be considered outliers putatively under directional or balancing selection. Simulations will be done iteratively to avoid an upward bias in quantile ranges (potentially resulting in Type I error for balancing selection) by removing outlier loci above the 95% quantile in the initial run as implemented in LOSITAN.

SNP Correlation and Association with Smoltification and Thermal Tolerance

In addition to identifying candidate loci as outliers from neutral expectations, allele frequencies in genes under directional selection may be directly correlated with selection gradients. We will test for correlation of minor allele frequencies (MAF) of SNP markers in each population versus specific trait characteristics of each reference population (i.e., potential for anadromy and thermal regime). Univariate regression analysis with the least squares method may be used to determine the relationship between MAF of each SNP and population parameters (e.g., temperature). In order to reduce false positives, alpha of 0.05 will be corrected for multiple tests across loci with the B-Y FDR method (Benjamini &Yekutieli 2001). Markers found to be significantly correlated with traits will be included in multiple regression analysis to create a regression model to predict thermal adaptation for each population. The subsequent values from the model will then be used to predict anadromy and thermal tolerance in natural populations throughout the Columbia Basin.

In order to limit association bias due to underlying population structure, analysis with STRUCTURE v.2.3.2 (Pritchard et al. 2000a; Hubisz et al. 2009) and STRAT v.1.1 (Pritchard et al. 2000b) will be implemented as suggested by Pritchard and Rosenberg (1999). In a recent review by Zhang et al. (2008), this approach has been shown to adequately account for stratification in association studies in comparison with other methods such as principal components analysis (e.g., Price et al. 2006). The procedure for running STRAT includes the following steps: 1) identify candidate loci with regression analysis, 2) remove candidate loci and any other significantly linked markers from the data set, 3) run STRUCTURE with remaining loci and select the most likely number of maximum populations ("k"), and 4) include ancestry coefficients ("Q values") and known traits to test candidate markers for significant association with traits. As needed, additional corrections for population structure will be included with outlier tests as discussed in Excoffier et al. 2009.

Population structure and Gene Flow with Neutral Markers

While candidate markers under selection can be used to address local adaptation in natural populations, the inclusion of neutral markers also provides the opportunity to evaluate gene flow among populations in relationship to environmental characteristics and geography. A variety of statistical models have been developed to address specific questions related to genetic structure due to environment and landscape features (reviewed in Manel et al. 2003; Storfer et al. 2006). For example, ordination models with canonical correspondence analysis have been used as an alternative to Mantel tests to simultaneously evaluate drainage, altitude, and human impacts to genetic diversity of salmonid fishes (Angers et al. 1999; Costello et al. 2003). Since many environmental features are inter-correlated, multivariate modeling of parameters related to genetic structure can be employed with tools such as GESTE (Foll and Gaggiotti 2006). Recent applications of interpolation models that utilize multivariate analyses such as principal components analysis (PCA) have also demonstrated that habitat and landscape features can identify and predict spatial patterns associated with restricted gene flow (Piertney et al. 1998). When PCA results are interpolated and overlaid with GIS data, synthesis maps can identify concordant genetic patterns related to landscape (e.g., Narum et al. 2008) as well as discordant patterns potentially related to local selection. In this study, we plan to apply these approaches to better understand environmental genetics of steelhead and Chinook salmon in the Columbia River.

Landscape genetics analyses methods will be utilized to evaluate hypotheses that landscape features influence neutral genetic structure as well as life history variation and local

adaptation of Chinook salmon and steelhead. Physical variables such as barriers, elevation, stream gradient, water temperature, and geographic distance from the ocean as RKM will be included as potential landscape features that influence genetic diversity of both selected and neutral markers. Genetic data will be obtained from existing baselines for Chinook salmon and steelhead in the Columbia River Basin, and additional tissues will be genotyped from populations with characteristics that are directly pertinent to the objectives of this study (e.g., populations with high/low thermal tolerance or residence/anadromy). Landscape features for genotyped populations will be determined using ArcGIS and simulations from PRISM. As appropriate, physical and genetic data will be analyzed following Narum et al. (2008) and new methods available in recent literature (e.g., Faubet and Gaggiotti 2008; Kalinowski et al. 2008; Joost et al. 2008).

Mantel tests will be utilized to test for genetic isolation by fluvial distance among sites. The regression of the pairwise $F_{ST}/(1- F_{ST})$ on geographic distance (GENEPOP; Raymond and Rousset 1995) will be used to determine significance of Mantel tests. Correlations between landscape features and heterozygosity will be tested with Spearman's *r*. A principle components analysis (PCA) will be completed with the R! statistical package (http://www.r-project.org/) and multivariate analysis in GESTE (Foll and Gaggiotti 2006) to determine which landscape features account for the majority of genetic diversity. Variables in the PCA include elevation, stream gradient, temperature, precipitation, and upstream distance from the ocean or other fixed points (as RKM). Values from PC1 and PC2 will be interpolated across the Columbia River Basin with the inverse distance squared method implemented by ArcGIS. The program STREAMTREE (Kalinowski et al. 2008) will be used to map genetic distances among populations. As possible, other analysis algorithms will also be utilized such as BIMr (Faubet and Gaggiotti 2008), GESTE (Foll and Gaggiotti 2006), and COLONISE (Foll and Gaggiotti 2005) to infer environmental as well as geological factors that influence vicariance events, migration and colonization in metapopulations. Potential introgression among genetic lineages or species will be evaluated with diagnostic SNP markers and analyzed with NEWHYBRIDS (Anderson and Thompson 2002).

Communication of Results
Results from this project will be included in annual reports and submitted for publication as warranted. Studies will also be presented at professional meetings and seminars as appropriate.

Objective 2) Controlled Experiments – Only *O. mykiss* in initial year(s)

Objective:
- We will explore gene expression and allele frequencies of candidate markers associated with phenotypic traits, beginning with smoltification and thermal tolerance.

Experimental Design
The first year of this study will begin to address two key traits related to recovery of steelhead, smoltification and thermal tolerance. Expression of genes related to these traits will be quantified in fish under controlled environments, and putative candidate genes for smoltification and thermal tolerance will be genotyped. Genetic markers for sex identification (Brunelli et al. 2008) will be utilized to verify sex of immature fish to analyze

sex-linked variation in phenotypes and candidate genes. Subsequent years of this project may include additional traits such as immune response and growth.

To evaluate smoltification, clonal lines of resident and anadromous strains of *O. mykiss* will be obtained from Gary Thorgaard's laboratory at Washington State University (WSU). Fry from WSU will be transferred to the Hagerman Fish Culture Experiment Station (HFCES) and reared under consistent conditions until they are approximately 10-20 grams (a size and age prior to smoltification) before experiments begin. The experiment will include controls and multiple treatments, with replicates of each as shown in Figure 1a.

For thermal tolerance, warm and cool adapted strains of *O. mykiss* will be obtained from both hatchery (WSU-Thorgaard Lab) and natural (desert and montane) collections. Fry from WSU will be transferred to HFCES and reared under consistent conditions until they are approximately 10-20 grams before experiments begin. Subsequent experiments may include larger fish (approximately 200 grams) to evaluate temperature effects on fish of differing size. Collections from natural populations will be completed in collaboration with biologists from Idaho Department of Fish & Game. In order to eliminate confounding effects from thermal history/acclimation of adults, gametes will be collected in the field from wild spawning adults. Adults will be collected with backpack shockers and gametes will be transported and fertilized at the HFCES. Eggs will be incubated near natal temperatures, and subsequent fry will be reared under consistent and equal conditions (i.e., experimental control temperature of 15 °C) until fish reach approximately 10-20 grams. The experiment will include controls and multiple treatments, with replicates of each as shown in Figure 1b.

Tissues will be sampled at multiple developmental stages from fish reared under controlled environments (temperature and photoperiod). The experiment will begin approximately 12 weeks after hatching, and samples will be collected at 3-4 time periods of development with up to 30 fish per sampling point (e.g., 3 replicate tanks of 10 fish, total of 90-120 fish). Fish will be sacrificed with an overdose of MS-222, and then immediately dissected to remove tissues such as brain, liver, gill, and fin for analysis. Tissues will be stored in RNALater and frozen for RNA extraction.

Specific candidate markers that have shown statistical evidence for selection in Objective 1 (i.e., with outlier and correlation tests) will be chosen for further evaluation in gene expression studies. For example, if glutamate dehydrogenase is indicated to be under differential directional selection among desert and montane populations of *O. mykiss*, the expression of this gene will be tested under controlled temperatures in a wet lab setting. Known genes that have been shown in previous studies to be involved with each trait (i.e., Na/K-ATPase for smoltification and HSP70 for thermal stress) will also be quantified as reference information for controlled experiments.

The expression of Na/K-ATPase genes is expected to change relative to photoperiod during smoltification due to ion regulation across the gills, and we will test for significant differential expression of this and other candidate genes (i.e., P53) over the course of fish development. Individual genotypes of candidate SNP markers and the allele frequencies of populations will also be tested for association with observed phenotypes and gene expression patterns. Candidate SNP markers that are validated in this manner will then be evaluated across the Columbia River Basin to determine potential of resident fish to contribute to anadromy in natural populations. Essentially, do resident populations contain smoltification

genes that can be expressed under the right environmental cues to trigger anadromy? Additional environmental triggers for anadromy / smoltification such as intermittent and seasonal flows will be evaluated in broad landscape models.

Gene expression of HSP70 is expected to vary in response to thermal stress (Iwama et al. 1998; Fowler et al. 2009). We will test for differential expression of HSP70 and other candidate genes (i.e., HSF2) and their association with survival under variable temperature conditions. Treatments will approach lethal temperatures and allow us to evaluate mortality of individual fish in these challenges. Individual genotypes and allele frequencies of candidate SNP markers will also be tested for association with observed phenotypes and gene expression patterns. This information will provide insight regarding genetic variability and the potential of fish to adapt to increasing water temperatures.

Quality Control

Positive and negative controls, standard quantification curves, and dissociation analysis will be utilized to assure quality of gene expression data. Genotype data for candidate markers will be checked for quality by utilizing positive and negative controls in each run. Repetitive genotyping of randomly selected individuals will be completed to ensure repeatability of genotyping results.

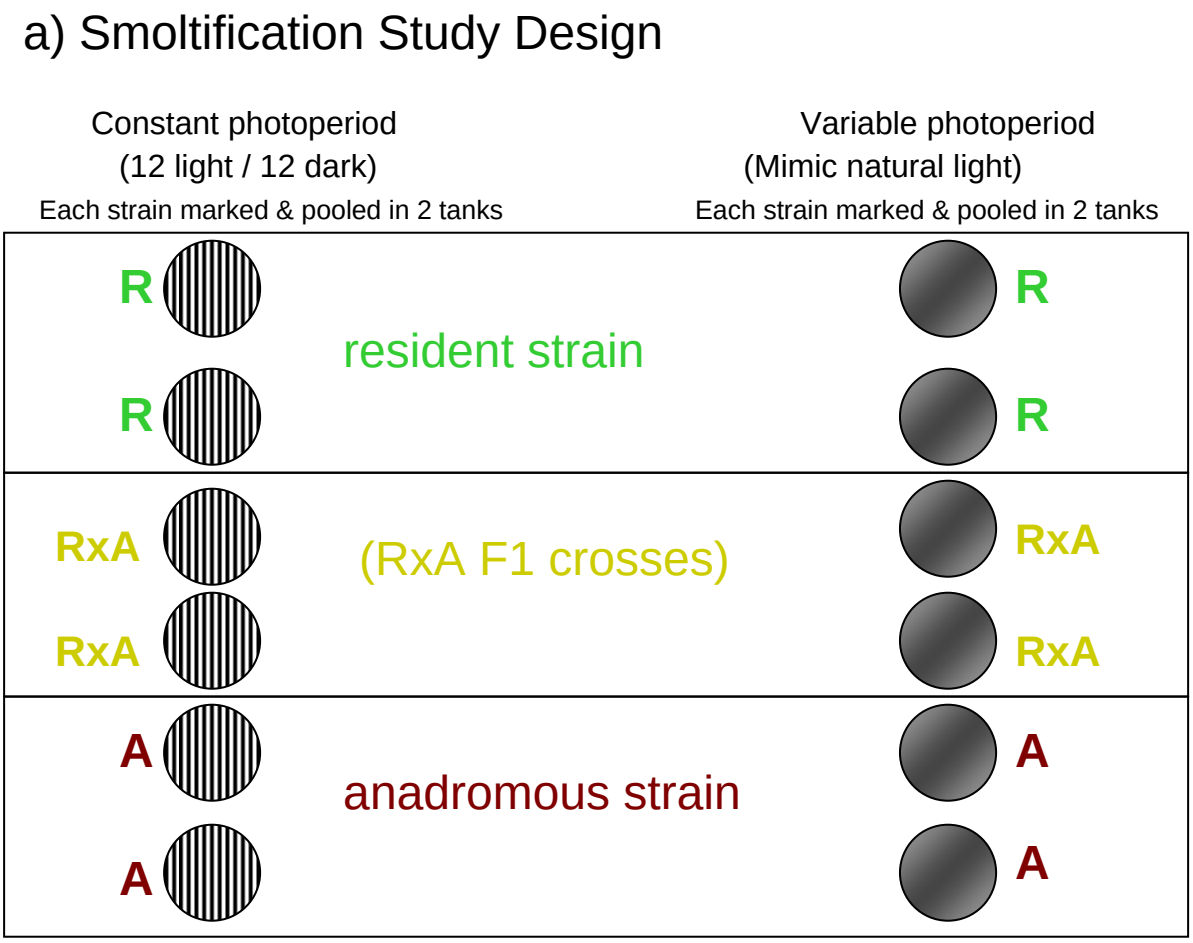
Statistical Analyses

Gene expression in tissues will be quantified relative to a standard curve of known amounts of cDNA, and normalized to a ubiquitous gene (i.e., “arp”). Gene expression among time periods and treatments will be tested for significant differences with t-tests and ANOVA. Allele frequencies will be estimated from candidate markers and tested for correlations with expression data and physical traits (i.e., condition factor, growth, survival).

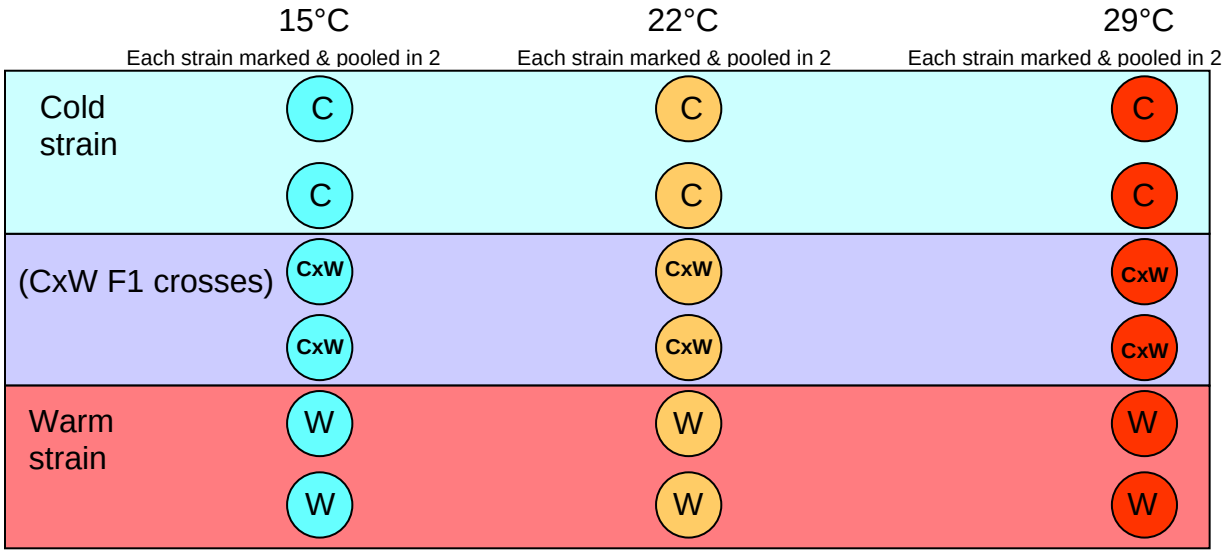
Communication of Results

Results from this project will be included in annual reports and submitted for publication as warranted. Studies will also be presented at professional meetings and seminars as appropriate.

Figure 1. Experimental design for a) smoltification study, and b) heat stress & thermal tolerance study.



b) Heat Stress Study Design



H. Facilities and equipment

Genetic analysis will be completed at the Hagerman Fish Culture Experiment Station (HFCES) in Hagerman, ID, operated by Columbia River Intertribal Fish Commission and University of Idaho staff. The Hagerman site houses multiple laboratories (including fish genetics, computing facilities, nutrition, and culture) and sufficient office space for the staff in this project. In addition to salaries, funding from this project will provide money for genetic laboratory supplies, wet lab supplies for fish culture, and field sampling gear. Existing equipment at HFCES will be sufficient to accommodate gene expression analysis and SNP genotyping for this study.

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J. Key personnel

The Lead Geneticist (Dr. Shawn Narum) will oversee this project and both of the objectives. Department Manager (Phil Roger) and Habitat Specialist/Senior Scientist (Dale McCullough) will facilitate coordination and administration of tasks related to the project. Key staff for completing objectives includes a Genomics Researcher (Nate Campbell), Conservation Geneticist (Andrew Matala), and a post-doctoral fellowship (TBD). Additional field and lab technicians will be also be important to completing objectives, but are not listed individually (see budget spreadsheet).

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Appointment

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Selected Publications

Narum, S.R., and N.R. Campbell. 2009. Sequence divergence of heat shock genes within and among three Oncorhynchids. Journal of Heredity doi:10.1093/jhered/esp081

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Selected Publications

Fang M, Li J, Blauwkamp T, Bhambhani C, **Campbell** N, Cadigan K (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. *EMBO Journal* **25**: 2735-2745

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2004-2008	Fishery Biologist/Geneticist, USFWS, Abernathy

Selected Publications

Microsatellite Variation Indicates Population Genetic Structure of Bocaccio. *North American Journal of Fisheries Management* 24:1189-1202, 2004.

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