United States Department of the Interior

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August 20, 2002

Columbia Basin Fish & Wildlife Authority
Attn: Executive Director
Within-Year Mod Request
2501 SW First Ave. Suite 200
Portland, OR 97201

Dear Director,

Enclosed please find a paper copy of my request for a within-year modification. As is explained in the text, this project was not funded in FY2002, but was originally scheduled for funding in FY2003.

If you have any questions, please contact me.

Sincerely,

Alec G. Maule, Ph.D.
Research Physiologist

Section 1. General administrative information

Title of project: 25052

BPA project number: Sex Reversal in Hanford Reach Fall Chinook Salmon

Project Sponsor requesting funding: USGS, Columbia River Research Laboratory

Business acronym (if appropriate): USGS, CRRL

Proposal contact person or principal investigator

Name	Alec G. Maule
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Project Leader: Alec G. Maule

Province: Columbia Plateau **Subbasin:** Mainstem Columbia

BPA Contracting Officer's Technical Representative: None – new project

Section 2. Description of within-year modification request

What is/are the target species? Fall Chinook Salmon

Location at which the action will be implemented (provide map). Is this the same study location that was identified in the original proposal? Hanford Reach. Yes, same as in original proposal.

Condition/situation creating the need for the modification. Is the situation the result of a catastrophic event that occurred within the existing study location and/or has a habitat/population/mechanical/structural dilemma developed in the proposed study location since the contract was signed with BPA?

This project was reviewed under the Columbia Plateau provincial review and funding was postponed until FY2003 awaiting completion of further work by one of our collaborators, Dr. Jim Nagler, University of Idaho. Nagler had previously reported that 80% of the spawned-out female fall chinook salmon in the Hanford Reach were genetic males based on a male-specific genetic marker (Nagler et al. 2001. Environmental Health Perspectives 109:67-69). Project 25052 is to examine this phenomenon further by looking for (1) physiological evidence of estrogen-specific pollution, (2) histological evidence of the simultaneous development of both ovaries and testes (intersexuality) in the same individual juvenile, (3) phenotypic sex ratio of

juvenile Hanford Reach fall chinook compared to that of fall chinook in the Little White Salmon National Fish Hatchery, and (4) genetic sex ratio using three male-specific genetic markers.

We now have results from several studies that support the need to initiate our project immediately. First, Jim Nagler has examined spawned-out females from the Hanford Reach and several other natural spawning populations in the Columbia River Basin and hatcheries. Nagler continues to find a significant proportion of phenotypic females testing positive for the male-specific genetic marker. To summarize by location, Nagler found the following percents of genetic males among spawning females in 2000: Hanford Reach 70%, Yakima River 18% and Ives Island 27%. Additionally, 38% of Hanford Reach phenotypic female fall chinook salmon were positive in 2001, and 98% and 62% of Priest Rapids Hatchery fish were positive in 2000 and 2001, respectively. Second, a soon-to-be published paper from the University of California, Davis reported similar research in the San Joaquin and Sacramento river basins. Williamson and May (Journal of Aquatic Animal Health, in press) examined fall chinook salmon females that spawned in seven streams and found 12 to 53% were genetic males (17% overall, 39 of 165), as compared to females in four hatcheries in the same streams that were 0 to 8% positive (3% overall, 3 of 88). Third, at a recent meeting (Salmon Recovery Symposium: Reproduction and Conservation; University of Idaho, Moscow, March 28-29, 2002; http://www.crb.wsu.edu/2FrontPages/Salmon.html) Dr. Robert Devlin, Fisheries and Oceans Canada, reported that he used two male-specific genetic markers—including the one used by Nagler, and Williamson and May—to screen 627 chinook salmon phenotypic females from 28 locations in British Columbia, the Yukon River, and Oregon. Two of the phenotypic females were positive for both male-specific markers, four were positive for only the marker used by Nagler and two were positive for only the second marker; thus, 1.3% of phenotypic females were positive for at least one male-specific marker.

I do not know the types of land that surround the streams from which Devlin sampled, but assuming that it is primarily forest and not much industry or agriculture, these three studies suggest a possible link between land use and reproductive dysfunction. Results from our project will help Columbia Basin fisheries managers determine whether or not there is need for concern. Therefore, I urge you to move this project forward for funding beginning in October 2002.

Provide explanations why the following alternative funding solutions are not feasible and will jeopardize the project

Reduction in existing scope of project	**Because the project is just beginning, a reduced scope will hinder our ability to address the objectives**
Deferral of existing tasks to a later date	The project has been delayed to FY2003

Identify the specific goal in the subbasin summary.

The Columbia Plateau Subbasin Summary for the mainstem Columbia River points out that the Tri-cities is the second largest urban area in the Columbia Basin, and that 47% of the lands in the Columbia Plateau are in agricultural production. Both of these factors would be expected to contribute to a high contaminant load in the Province. In fact, the USGS has reported

(Williamson et. al. 1998) that surface water quality in the Central Columbia plateau is significantly impaired due to urban and agricultural practices. Water quality problems cited by the USGS include; eutrophication, sediment erosion, and high pesticide concentrations, levels of which exceed criteria for the protection of aquatic life at some sites. The Subbasin Summary goes on to describe concerns about contaminants, and emphasizes the need to develop indicators of ecosystem health in the Hanford Reach and to assess the possible impacts of Hanford site development activities on the aquatic ecosystem.

What existing objective (in the subbasin summary as well the existing proposal) does the proposed modification address? If the request is for a modification to an existing task, identify the task that is being modified.

Objective	Existing Task	New Task
All objectives from original proposal need to be addressed. Please see next section for Objectives and methods.		

Proposed action to achieve the objective (detailed description of the task/methods, associated monitoring and evaluation, and schedule for completion)

Objective 1 - Determine the levels of biomarkers of estrogen-specific pollution, and the activity of physiological indicators of contaminant exposure in Hanford Reach juvenile fall chinook salmon.

Question of interest: Do fish collected from the Hanford Reach or a non-contaminated reference site exhibit biomarker levels indicative of xenoestrogen or contaminant exposure?

Task 1a- Collect and sample 200 juvenile fall chinook salmon from the Hanford Reach and 200 up-river bright fall chinook salmon from the Little White Salmon National Fish Hatchery (LWSNFH).

Task 1b- Determine the levels of the estrogen responsive biomarkers Vg and zrp in Hanford Reach and LWSNFH juvenile fall chinook salmon.

Task 1c- Determine the activity of total cytochrome P-450 (P-450) and ethoxyresofurin O-deethylase (EROD) enzymes in the liver of Hanford Reach juvenile fall chinook salmon.

Task 1d- Determine the levels of malondialdehyde (an indicator of lipid peroxidation) in liver preparations from Hanford Reach juvenile fall chinook salmon

Methods

Juvenile fall chinook salmon will be collected from the Hanford Reach using a combination of beach seining and electrofishing in the spring (May - mid June) of 2002. A sample size of 200 fish will be collected from the Hanford Reach and a reference site (total $N = 400$). Sample size was determined to allow for a minimum detectable difference of 0.15 in a comparison (t-test) between mean biomarker levels in Hanford Reach fish and fish from a reference site (Zar 1984). A beta level of 0.1 (statistical power = 0.9) and an alpha level of 0.05 were used in this analysis, and an estimated variance of 0.4 was based on data presented in Arukwe et al. (2000). In the Hanford Reach, sampling will occur at the spawning areas of Vernita Bar and Lock Island (Dauble and Watson 1997). Additional sites, if needed, will be selected to cover the range of

habitat and area in the Hanford Reach. As a reference site having an uncontaminated population, we will sample "upriver bright" juvenile fall chinook salmon from the Little White Salmon National Fish Hatchery (LWSNFH; Washington). Plasma, liver and gonad samples will be collected from all fish. Because of their potentially small size, some samples may have to be pooled for analysis. Plasma Vg and zrp will be measured using an indirect ELISA technique (Arukwe et. al. 1997). Antibodies for the measurement of Vg and zrp will be obtained from Biosense Laboratories (Bergen, Norway). Livers will be processed and assayed for total cytochrome P-450 activity, EROD activity, and levels of malondialdehyde using methods described in Estabrook et al. (1972), Prough et al. (1978), Burke and Mayer (1974), Carpenter et al. (1990), and Plaa and Witschi (1960). For specifics concerning these assays, we will receive training from Dr. Gene Foster, Oregon Department of Environment Quality. Gonad samples will be fixed in buffered formalin for histological processing and analysis. For all samples, we will calculate means and standard errors and compare them between sites using a *t*-test or a non-parametric equivalent.

Objective 2 – Determine the incidence of intersexuality in Hanford Reach juvenile fall chinook salmon by histological analysis of gonadal tissue.
Question of Interest: Do Hanford Reach or LWSNFH fish exhibit abnormal gonadal morphology indicative of chemical exposure?

Task 2a- Gonad samples from juvenile fall chinook (collected in Obj. 1) will be processed using standard histological techniques. Ovarian and testicular morphology will be assessed and the phenotypic sex ratio and incidence of intersexuality determined.

Methods

Gonad samples obtained as part of Objective 1 will be used. Both gonads from each fish will be serially sectioned (3-5 μm) in a longitudinal orientation and every 5^{th} section mounted on glass microscope slides. The tissues will be stained with hematoxylin and eosin, and coverslipped. The Histology Core Laboratory of the WSU-UI Center for Reproductive Biology will conduct all tissue processing, sectioning, mounting and staining. Gonadal morphology will be assessed via light microscopy to document any intersex condition and determine the phenotypic sex ratio as previously described (Krisfalusi and Nagler 2000). Sex ratio data and the prevalence of intersex gonads will be compared between sites using Chi square analysis.

Objective 3- Determine the phenotypic sex ratio of Hanford Reach or LWSNFH juvenile fall chinook salmon by examination of the gonads.
Question of Interest: Does the phenotypic sex ratio of the juvenile fish population in the Hanford Reach differ significantly from the expected 1:1 ratio?

Task 3a- Collect 750 juvenile fall chinook salmon from the Hanford Reach and 750 up-river bright fall chinook salmon from the LWSNFH.
Task 3c- Raise Hanford Reach and LWSNFH juvenile fall chinook salmon and sample them when they are at least 120 mm fork length.
Task 3b- Determine the gonadal sex of Hanford Reach and LWSNFH juvenile fall chinook salmon.

Methods

Juvenile fall chinook salmon will be collected (mid-May to Mid-June, 2002) from the Hanford Reach using a combination of beach seining and electrofishing. A minimum sample size of 750 fish will be collected from the Hanford Reach and LWSNFH. To provide representative coverage of the Hanford Reach, we will sample several (up to six) areas, especially targeting the main spawning areas of Vernita Bar and Lock Island. Sample size was determined to allow for a minimum detectable difference of 10% when comparing (using Fisher's exact test) the prevalence of females between the two sites (Zar 1984). An alpha level of 0.05 and a beta level of 0.1 were used in this determination. Weight and total length data will be recorded for all fish. Juvenile fall chinook will be collected, transported to the CRRL, and maintained until they reach an adequate size for gross determination of gonadal sex. To ensure that transport and culture of these fish in the laboratory does not affect the determination of sex, sample collection will occur in late May, which should be well past the point of susceptibility to chemical induced sex reversal. Although determination of sex would be possible immediately after collection, this period of growth in the laboratory will allow us to more easily determine gonadal sex and may allow us to detect gonadal anomalies that might otherwise go undetected if fish were examined at a smaller size. Fish will be held in fiberglass tanks (1.2-m-diameter) supplied with flow through well water and fed a commercial diet and bloodworms twice daily. When an average total length of greater than 120 mm is reached, fish will be killed and preserved in buffered formalin for later inspection of the gonads. Gonadal sex will be determined by visual inspection of the gonads using a Wild MZ6 dissecting microscope. An aceto-carmine stain will be used to help visualize gonadal structure. The proportion of females present in the Hanford Reach will be compared to both a theoretical 1:1 ratio and also the proportion of females observed at the reference site using Fisher's exact test (Zar 1984).

Objective 4 - Determine the genotypic sex ratio of Hanford reach juvenile fall chinook salmon. Question of Interest: Does the genotypic sex ratio of Hanford Reach or LWSNFH fall chinook differ from either the phenotypic sex ratio or a theoretical 1:1 ratio?

Task 4a- Determine the genetic sex ratio in juvenile fall chinook from the Hanford Reach and the LWSNFH.

Methods

Three different male specific markers, one developed by Devlin et al (1991, 1994), a growth hormone pseudogene marker (Du et al. 1993), and a marker developed by Clifton and Rodriguez (1997) will be used. Tissue samples (caudal fin and peduncle) will be obtained from all fish sampled for Objective 1 and fixed in 95% ethanol. Genomic DNA will be isolated from 5-10 mg of the fixed tissue, using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). A protocol for fixed solid tissues supplied by the vendor will be followed. The genomic DNA obtained will be stored in DNA Hydration Solution (Tris-EDTA buffer) at –80°C until used. Polymerase chain reactions (PCRs) will be performed with genomic DNA using reagents supplied in the Taq DNA Polymerase kit (GibcoBRL, Rockville, MD). The University of Idaho laboratory will use two different PCR primer sets that amplify male-specific DNA sequences in

chinook salmon, specifically we propose to use the OTY1 (Devlin et al. 1994) and the salmon pseudogene (Du et al. 1993) genetic markers. Polymerase chain reaction tests for these genetic markers amplify discreet regions on the Y-chromosome (i.e., qualitative) and are therefore male specific (since female chinook salmon have two X sex chromosomes). Following PCR, the DNA products will be electrophoresed under standard conditions in agarose gels containing ethidium bromide. The patterns of the ethidium bromide-stained DNA are visualized on an ultraviolet light table and digitally recorded on a computerized analysis system. This technique will permit the screening of each fish to determine the genetic sex, which will then be compared to the expressed phenotype of each individual as determined by histological analysis in Objective 2. The use of different genetic tests on each fish will reduce the possibility of obtaining false positives.

The USGS Western Fisheries Research Center (WFRC) will also analyze DNA extractions using the marker OT24 that quantitatively discriminates sex in chinook salmon (Clifton and Rodriguez, 1997). The OT24 is a quantitative marker that occurs approximately 100 times more frequently in males than females. Primers for PCR have been designed that specifically amplify OT24. Since OT24 is quantitative, genomic DNA extracted from fin samples will be quantitated by fluorescence and an equal amount of DNA PCR amplified. To avoid false negative results, the OT24 primers will be multiplexed with primers that specifically amplify the hsp30 gene in chinook salmon as previously described (Clifton and Rodriguez, 1997). Results from these markers will be compared against each other as well as against a theoretical 1:1 ratio and the phenotypic results from Objectives 2 and 3

If applicable, identify the Reasonable and Prudent Alternatives (RPAs)/RPMs or Biological Opinion (BiOp) Actions that the request addresses and explain how implementing the proposed actions will result in addressing the RPA or BiOp action.

RPA or BiOp Action	
RPA 150	This work will determine if the habitat in the Hanford Reach is at risk of degradation.
RPA 155	This project will identify research needs in mainstem habitat, and begin to address cause-and-effect relations between habitat water quality and reproduction.
RPA 182	This project will address differences in reproductive success between hatchery and wild chinook salmon.

Section 3. Milestones completed

Year	Work initiated/completed
	The project is scheduled to begin in FY2003

Section 4. Relationships to other projects in this basin

Project #	Title/description	Nature of relationship

Innovative #22013	Genetic sex of chinook salmon in the Columbia River Basin	Although the PI of this project (J. Nagler) is a collaborator on the new project, and we are addressing the same question, the projects are administratively independent.

Does not implementing this modification jeopardize the ability to achieve existing biological objectives of this project as well as other projects (explain)? Yes. As the project has not yet started, none of the objectives have been met.

Section 5. Budget for Planning & Design phase

Task-based budget

Objective	Task	Duration in FYs	Estimated FY cost	Subcontractor
		Total		

Out year objective-based budget

Objective	Starting FY	Ending FY	Estimated cost

Out year budgets

FY	FY	FY	FY

Section 6. Budget for Construction/Implementation phase

Task-based budget

Objective	Task	Duration in FYs	Estimated FY 2003 cost	Subcontractor
Objective 1. (please see above for descriptions of objectives & tasks)	Task 1a	FY03	$35,887	
	Task 1b	FY03-04	28,678	
	Task 1c	FY03-04	22,363	
	Task 1d	FY03-04	19,004	
Objective 2.	Task 2a	FY03-04	25,521	$ 5,920
Objective 3.	Task 3a	FY03	38,744	
	Task 3b	FY03	18,420	
	Task 3c	FY03-04	17,468	
Objective 4.	Task 4a	FY03-04	60,404	$29,600

Total	**$266,489**	**$35,520**

Out year objective-based budget

Objective	Starting FY	Ending FY	Estimated cost
Objective 1	2003	2004	$85,881
Objective 2	2003	2004	27,969
Objective 3	2003	2004	38,737
Objective 4	2003	2004	6,174

Out year budgets

FY 2004	FY	FY	FY
$158,762			

Section 7. Budget for O & M phase

Task-based budget

Objective	Task	Duration in FYs	Estimated FY 2003 cost	Subcontractor
		Total		

Out year objective-based budget

Objective	Starting FY	Ending FY	Estimated cost

Out year budgets

FY	FY	FY	FY

Section 8. Budget for M & E phase

Task-based budget

Objective	Task	Duration in FYs	Estimated FY 2003 cost	Subcontractor
		Total		

Out year objective-based budget

Objective	Starting FY	Ending FY	Estimated cost

Out year budgets

FY	FY	FY	FY

Section 9. Estimated budget summary

Itemized budget

Item	Note	FY03 + 04
Personnel		$167,006
Fringe		50,102
Supplies		39,500
Travel		22,669
Subcontractor		39,270
Capital Equipment		
Overhead/G-A Costs		106,711
Other		
	Total	425,251

Other funds needed to complete this modification

Organization	Item or service provided	Amount	Cash or in-kind
	Total cost-share		

Total estimated request

Total FY 2002 request	0

Effects on outyear

	FY 2003	FY 2004	FY 2005	FY 2006
Planning and design				
Construction/implementation	$266,489	$158,762		
Operations and maintenance				
Monitoring and evaluation				
Total outyear budgets				

Reference (include web address if available online)

Arukwe, A., Knudsen, F.R., and Goksøyr, A., 1997. Fish zona radiata protein: a sensitive biomarker for environmental estrogens. Environ. Health Perspect. 105:418-422.

Arukwe, A., Celius, T., Walther, B., and Goksøyr, A., 2000. Effects of xenoestrogen treatment on *zona radiata* protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 49:159-170.

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Clifton, D. R., and Rodriguez, R.J., 1997. Characterization and application of a quantitative DNA marker that discriminates sex in chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci. 54:2647-2652.

Dauble, D.D., and Watson, D.G., 1997. Status of fall chinook salmon populations in the Mid-Columbia River, 1948-1992. N. Amer. J. Fish. Mgmt. 17:283-300.

Devlin, R.H., McNeil, B.K., Groves, T.D.D., and Donaldson, E.M., 1991. Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci. 48:1606-1612.

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Reference (include web address if available online)
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Krisfalusi, M., and Nagler, J.J., 2000. Induction of gonadal intersex in genotypic male rainbow trout (*Oncorhynchus mykiss*) embryos following immersion in estradiol-17β. Mol. Reprod. Develop. 56: 495-501
Nagler, J.J., Bouma, J., Thorgaard, G.H., and Dauble, D.D., 2001. High incidence of a male-specific genetic marker in phenotypic female chinook salmon from the Columbia river. Environ. Health Perspect. 109:67-69.
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