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## PART I - ADMINISTRATIVE

### Section 1. General administrative information

Title of project

Preserve Listed Salmonid Stocks Gametes

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**BPA project number:** 9703800  
**Contract renewal date (mm/yyyy):** 1/2000  **Multiple actions?**

**Business name of agency, institution or organization requesting funding**  
Nez Perce Tribe Department of Fisheries Resources Management

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**Business acronym (if appropriate)** NPT

**Proposal contact person or principal investigator:**

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**NPPC Program Measure Number(s) which this project addresses**  
7.4E, 7.4D, 7.2D

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**FWS/NMFS Biological Opinion Number(s) which this project addresses**  
ESA Section 10 permit #1134

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**Other planning document references**

Snake River Salmon Recovery Plan (NMFS 1994); IV.A The recovery goal.....is to restore these distinct populations (and their genetic and demographic subunits) .....

IV.A.5 Objectives supporting the recovery goal - Judiciously use hatchery production..... but exercise caution to avoid introductions which can degrade the genomes of natural stocks. a. Supplement the weakened natural stock with hatchery propagated fish, but only of the same genetic lineage.

IV.C.6 The following principles have influenced team evaluations and decisions....and should also serve as guidelines.... a.) Biological Diversity - The biological diversity of the listed species must be maintained, and particular attention must be paid to the array of genomes .... 2) some 38 separate breeding subpopulations.....

Strategy for Salmon (NWPPC 1992) calls for research improvements in cryopreservation technology, and development applications to preserve salmon eggs for future use.

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**Short description**

Establish a gene bank to preserve male gametes from listed steelhead and chinook salmon conservation units that are at low levels of abundance and at high risk of extirpation.

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**Target species**

Steelhead and spring and summer chinook salmon

**Section 2. Sorting and evaluation**

**Subbasin**

Salmon, Clearater, Grande Ronde, Imnaha, Tucannon, and Lower Snake tributaries

**Evaluation Process Sort**

CBFWA caucus	Special evaluation process	ISRP project type
Mark one or more caucus	If your project fits either of these processes, mark one or both	Mark one or more categories
<input checked="" type="checkbox"/> Anadromous fish <input type="checkbox"/> Resident fish <input type="checkbox"/> Wildlife	<input checked="" type="checkbox"/> Multi-year (milestone-based evaluation) <input type="checkbox"/> Watershed project evaluation	<input type="checkbox"/> Watershed councils/model watersheds <input type="checkbox"/> Information dissemination <input type="checkbox"/> Operation & maintenance <input type="checkbox"/> New construction <input checked="" type="checkbox"/> Research & monitoring <input checked="" type="checkbox"/> Implementation & management <input type="checkbox"/> Wildlife habitat acquisitions

**Section 3. Relationships to other Bonneville projects**

**Umbrella / sub-proposal relationships.** List umbrella project first.

Project #	Project title/description

**Other dependent or critically-related projects**

Project #	Project title/description	Nature of relationship
9604300	Johnson Creek Artificial Propagation Enhancement Project	Preserved genetic material may be used in spawning protocols to promote genetic diversity.
	Lower Snake River Compensation Plan Hatchery Production	Preserved genetic material may be used in spawning protocols to promote genetic diversity.
960440	Grande Ronde Spring Chinook Captive Broodstock Project	Preserved genetic material may be used in spawning protocols to promote genetic diversity.
9800702	Grande Ronde Supplementation - Lostine River	Preserved genetic material may be used in spawning protocols to promote genetic diversity.
	Idaho Department of Fish and Game Rapid River Hatchery	Preserved genetic material may be used in spawning protocols to promote genetic diversity.
	Intracytoplasmic Sperm Injection (ICSI):Genetic Retrieval from Single Sperm	Develop methodology to produce viable fish (salmonids) by injecting a single sperm nucleus into the blastodisc (activated egg).

## Section 4. Objectives, tasks and schedules

### *Past accomplishments*

Year	Accomplishment	Met biological objectives?
1997	Cryopreserved 189 chinook salmon samples	Yes - objectives 2a,b and 3a,b,c,d,e were met. Objective 3c was not met.
1998	Finalized and submitted 1997 annual report to BPA	Yes - objective 4a was met.
1998	Cryopreserved 296 chinook salmon samples	Yes - objectives 2a,b and 3a,b,c,d,e were met. Objective 3c was not met.
1998	Conducted fertilization trials with cryopreserved semen versus fresh semen at Washington State University	Yes - objective 3b was met.
1998	Thawed cryopreserved semen and fertilized Grande Ronde basin chinook captive broodstock eggs	Yes - objective 3b was met.
1998	Cryopreserved 101 Grande Ronde Basin captive broodstock chinook salmon male gametes	Yes - objectives 2a,b and 3b,e were met. Objective 3c was not met.

### *Objectives and tasks*

Obj 1,2,3	Objective	Task a,b,c	Task
1	Coordinate the listed salmonid stocks gamete preservation with management agencies and Tribes.	a	Identify and meet with other agencies and Tribes who may help with the collection of gametes or potentially use the cryopreserved samples.
		b	Keep current of the best cryogenic technology.
		c	Provide annual reports to NMFS which summarizes project activities relating listed salmonids under ESA
2	Define cryopreservation project goals for gene banking of gametes from chinook salmon and steelhead populations at high risk of extirpation in the Snake River basin.	a	Identify chinook salmon and steelhead conservation units (populations) that are at high risk of extirpation in the Snake River basin.
		b	Determine chinook salmon populations for germ plasm sampling.
		c	Determine sample sizes required per population to preserve a representative sample of the genetic diversity within a population.
		d	Determine the period of time (years) for cryopreservation to occur, per population, to ensure that sufficient directly non-related

			individual genetic material is preserved.
3	Apply cryopreservation techniques to chinook salmon conservation units at low levels of abundance and high risk of extirpation.	a	Apply for needed ESA Section 10 research permits for cryopreservation purposes.
		b	Develop and utilize detailed adult sampling protocols and cryopreservation techniques for the collection, preservation, storage and inventory of male salmon germ plasm.
		c	Cryopreserve 100 adult male chinook salmon and steelhead gametes each year from each conservation units identified as being at low levels of abundance and high risk of extirpation.
		d	Establish gene bank locations in at least two independent locations.
		e	Preserve gamete samples on-site or at the identified independent locations.
		f	Conduct genetic analysis on fish from which semen was cryopreserved.
4	Support research on the cryopreservation of female gametes.	a	Fund/support new cryogenic technology to preserve the female complement of genetic material.
5	Transfer of Technology	a	Prepare and provide quarterly and annual reports summarizing all activities associated with cryopreservation sample collection, preservation and storage.
		b	Educate agencies, Tribes, and the public about the salmonid gamete preservation program.

### **Objective schedules and costs**

<b>Obj #</b>	<b>Start date mm/yyyy</b>	<b>End date mm/yyyy</b>	<b>Measureable biological objective(s)</b>	<b>Milestone</b>	<b>FY2000 Cost %</b>
1	7/1997	6/2004		NMFS annual report	10.00%
2	7/1997	12/2004	Develop and update sampling methodology	Finalized written protocol	5.00%
3	7/1997	3/2005	565 chinook salmon cryopreserved samples, 25 steelhead cryopreserved samples	100 samples per species per year per spawning aggregate	50.00%
4	1/2000	12/2003	Preserving female genetic material.	Successful cryopreservation of female genetic material.	10.00%
5	8/1997	4/2005	1997 annual report submitted to BPA	BPA annual report	25.00%
				<b>Total</b>	100.00%

### **Schedule constraints**

Annual abundance in salmonid populations will determine if sufficient samples can be gene banked within the prescribed duration of the project. Sample timing must be closely coordinated with surveyors to ensure only spawned-out males are available.

**Completion date**

>2005. The project should be evaluated in 2002. Attention will then be focused on populations with low natural returns. Operation and maintenance will need to continue on a reduced budget.

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**Section 5. Budget**

**FY99 project budget (BPA obligated):** \$160,000

***FY2000 budget by line item***

<b>Item</b>	<b>Note</b>	<b>% of total</b>	<b>FY2000</b>
Personnel	Project Leader, Program Manager, Office Manager, Technicians, Admin. support	%33	61,000
Fringe benefits	27% permanent employees	%9	16,540
Supplies, materials, non-expendable property	field supplies, liquid nitrogen, computer lease	%4	8,000
Operations & maintenance	office services, training	%6	12,000
Capital acquisitions or improvements (e.g. land, buildings, major equip.)		%0	
NEPA costs		%0	
Construction-related support		%0	
PIT tags	# of tags:	%0	
Travel	Air transport of cryopreserved samples, per diem, vehicle, admin support and project leader travel	%11	20,000
Indirect costs	22.9% of above direct costs	%14	26,582
Subcontractor	cryopreservation assistance from Washington State University	%4	7,000
Subcontractor	cryoreservation assistance from University of Idaho and support of vitrification of embryos research	%15	27,000
Subcontractor	University of Idaho Fish Genetics Laboratory genetic analysis	%4	7,000
Other		%0	
<b>TOTAL BPA FY2000 BUDGET REQUEST</b>			<b>\$185,122</b>

***Cost sharing***

<b>Organization</b>	<b>Item or service provided</b>	<b>% total project cost (incl. BPA)</b>	<b>Amount (\$)</b>
USFWS/LSRCP	LSRCP salary and fringe benefits	%5	9,550
	Vehicle and Mileage	%1	1,500
	Perdiem	%1	1,110
	Field Supplies	%0	500
	Indirect Rate	%1	2,760
<b>Total project cost (including BPA portion)</b>			<b>\$200,542</b>

## Outyear costs

	FY2001	FY02	FY03	FY04
<b>Total budget</b>	\$180,000	\$182,000	\$184,000	\$186,000

## Section 6. References

Watershed?	Reference
<input type="checkbox"/>	Allendorf, F. W. and N. Ryman. 1987. Genetic Management of Hatchery Stocks. In "Population Genetics and Fishery Management". University of Washington Press, Seattle, Washington.
<input type="checkbox"/>	Cloud, J. G. 1998. Personal Communication, Department of Biological Sciences, University of Idaho. Moscow, Idaho.
<input type="checkbox"/>	Cloud, J. G., W. H. Miller and M. J. Levanduski. 1990. Cryopreservation of salmon sperm as a means to transfer genes from wild fish to hatchery populations: A field evaluation. Progressive Fish Culturist. 52:51-53.
<input type="checkbox"/>	Cloud, J. G. and C. Osborne. 1997. Cryopreservation of salmonid sperm. Department of Biological Sciences, University of Idaho. Moscow, Idaho.
<input type="checkbox"/>	Columbia River Basin Fish and Wildlife Program. 1994. Northwest Power Planning Council. Portland, Oregon.
<input type="checkbox"/>	Fahy, G. M. 1986. Vitrification: A new approach to organ cryopreservation. Progressive Clinical Biological Research. 224:305-335.
<input type="checkbox"/>	Faurot, D., R. D. Armstrong, P. A. Kucera, and M. L. Blenden. 1998. Cryopreservation of adult male spring and summer chinook salmon gametes in the Snake River basin. BPA Annual Report. Portland, Oregon.
<input type="checkbox"/>	Ferguson, F. 1995. The role of molecular genetic markers in the management of cultured fishes. In Molecular Genetics in Fisheries (Carvalho, G.R. and T. L. Pitcher, editors). Chapman and Hall, London. pp 81-103.
<input type="checkbox"/>	Hagedorn, M., F. W. Kleinhans, D. E. Wildt, and W. F. Rall. 1997. Chill sensitivity and cryoprotectant permeability in dechorionated zebrafish embryos, Brachydanio rerio. Cryobiology. 34:251-263.
<input type="checkbox"/>	Hagedorn, M., F. W. Kleinhans, D. Artemov and U. Pilatus. 1998. Characterization of a major permeability barrier in the zebrafish embryo. Biol. Reprod. 59:1240-1250.
<input type="checkbox"/>	Grande Ronde Basin Captive Broodstock Status Update and Annual Operating Plan. 1998. Oregon Department of Fish and Wildlife, Confederated Tribes of the Umatilla Indian Nation, and Nez Perce Tribe.
<input type="checkbox"/>	Mounib, M. S. 1978. Cryogenic preservation of fish and mammalian spermatozoa. Journal of Reproductivity Fertilization. 53:13-18.
<input type="checkbox"/>	Nilsson, E. E. and J. G. Cloud. 1993. Cryopreservation of rainbow trout (Oncorhynchus mykiss) blastomeres. Aquatic Living Res. 6:77-80.
<input type="checkbox"/>	Powell, M. 1998. Personal Communication. Hagerman Fish Culture Experiment Station, Hagerman, Idaho.
<input type="checkbox"/>	Rall, W. F. and G. M. Frey. 1985. Ice-free cryopreservation of mouse embryos at -196 C by vitrification. Nature. 313:573-573-575.
<input type="checkbox"/>	Snake River Recovery Plan. 1994. National Marine Fisheries Service. Seattle, Washington.
<input type="checkbox"/>	Strategy for Salmon. 1992. Northwest Power Planning Council. Portland, Oregon.
<input type="checkbox"/>	Suzuki, T., H. Kimada, R. Takai, K. Arie and T. T. Kozima. 1995. Relation between toxicity of cryoprotectant DMSO and its concentration in several fish embryos. Fish Science. 61:193-197.
<input type="checkbox"/>	Thorgaard, G. H. and J. G. Cloud. 1993. Reconstitution of genetic strains of salmonids using biotechnical approaches. In "Genetic Conservation of Salmonid Fishes". (J. G. Cloud and G. H. Thorgaard, editors) Plenum Press, New York, NY.
<input checked="" type="checkbox"/>	Thorgaard, G. H., P. A. Wheeler and J. G. Cloud. 1998. Status and potential value of sperm banking for Snake River salmon. Proceedings of the Columbia River Anadromous Salmonid

	Rehabilitation and Passage Symposium (E. L. Brannon and W.C. Kinsel, editors).
<input type="checkbox"/>	Wy-Kan-Ush-Mi Wa-Kish-Wit (Spirit of the Salmon). 1995. Columbia River Inter-tribal Fish Commission. Portland, Oregon.
<input type="checkbox"/>	Zhang, T., and D.M. Rawson. 1995. Studies on chilling sensitivities of zebrafish ( <i>Brachydanio rerio</i> ) embryos. <i>Cryobiology</i> . 32:239-246.

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## PART II - NARRATIVE

### Section 7. Abstract

Snake River steelhead (*Oncorhynchus mykiss*) and spring and summer chinook salmon (*Oncorhynchus tshawytscha*) populations have experienced significant decline in population numbers over the past five decades and are now a listed species under the ESA. Genetic conservation through population protection has not been successful. The threat of losing genetic diversity in native fish stocks dictates the establishment of long-term storage of fish germ plasm to serve as insurance for ongoing conservation programs. A genetic resource management approach using cryogenic techniques is recommended. The goal of the Listed Salmonid Stocks Gamete Preservation project seeks to apply cryogenic technology to preserve the genetic diversity of Snake River steelhead and chinook salmon spawning aggregates that are at low levels of abundance and high risk of extirpation. This approach would target steelhead and chinook salmon populations with ongoing conventional hatchery or captive brood stock artificial propagation programs, to preserve and use cryopreserved material to enhance genetic diversity. Secondly, this project will establish long-term germ plasm repositories at the University of Idaho and Washington State University as an insurance policy in case extirpation of steelhead and chinook salmon populations does occur. At least five years of collection and 100 cryopreserved samples per year are estimated to be needed per spawning aggregate per species to ensure that sufficient non-related individual genetic material is preserved. When used with hatchery propagation, collected sperm could enhance genetic diversity of the propagated population. When gene banked at repositories, samples preserved would allow for future management and research options. Results will be summarized in quarterly and annual reports to BPA.

### Section 8. Project description

#### a. Technical and/or scientific background

Snake River steelhead and spring/summer chinook salmon populations have experienced significant decline in population numbers over the past five decades and are now listed as a threatened species under the Endangered Species Act. Genetic conservation through population protection has not been successful.

In order to reduce or reverse the declines in fish populations, fish hatcheries have been established to mitigate for the construction of dams and associated loss of anadromous fish, loss of spawning and rearing habitat and to enhance the reproductive output of fish stocks. Although hatcheries have been an important tool in the enhancement of fish populations, they have some inherent weaknesses relative to the maintenance of the original genetic composition of fish stocks (Cloud 1998). Wild and natural salmon and steelhead populations are a crucial source of genetic variability for sustaining the hatchery populations.

The threat of losing genetic diversity in specific native fish stocks has led to the establishment of a program for the long-term storage of fish germ plasm to serve as an insurance for ongoing conservation programs. Gene banking is one way to ensure that representative genetic samples from the original population is preserved and available in a germ plasm repository now, before the population is extinct. Presently, cryopreservation (long-term storage of material at extremely low temperatures) of male gametes (semen) is the only functional means of storing fish germ plasm for extended periods of time. Successful research and development in the technology for preservation of female gametes would allow the ability to

preserve germ plasm components from male and female chinook salmon to preserve future management options.

There are two important factors to be considered when establishing a germ plasm repository. First, this is a genetic repository, and will not solve population problems of a stock that is decreasing. Secondly, fertility of the frozen/thawed gametes will not be greater than the fertility of the starting material. The quality of thawed sperm is usually a direct reflection of the quality of the sperm that was cryopreserved, and 50-80% motility of sperm is considered good.

We would desire that fertilization rates using cryopreserved semen would average 80% or higher when used in conventional hatchery programs. There is a risk of lower fertilization rates and potential loss of eggs using cryopreserved semen. Lesser fertilization rates may be acceptable where genetic concerns warrant them, such as in captive brood stock programs. The section 10 permit #1134 allows for collection of spawned-out males from the spawning grounds. This assumes that sufficient quantity and quality of semen can be obtained from spawned-out males for gene banking purposes.

In 1992, cryopreservation of adult male spring and summer chinook salmon gametes was initiated by the Nez Perce Tribe through funding by the U.S. Fish and Wildlife Service in cooperation with the Lower Snake River Compensation Plan hatchery evaluations program. Cryopreservation activities continued through 1996 on selected salmon spawning aggregates. Since funding was minimal, a total of 80 samples were collected and preserved from 1992-1996 from four river subbasins in the Snake River basin. A small annual cost-share from the Tribal LSRCP hatchery evaluations project continues for assistance in collection of spawned-out male salmon over the wide geographic expanse of tributary streams in the Snake River basin. Assistance also comes from LSRCP staff in the Enterprise, Oregon, field office to collect samples from Lookingglass Fish Hatchery, Little Sheep Creek and Rapid River Hatchery.

The project was fully funded by BPA in 1997. In 1997, 198 viable cryopreservation samples were taken from nine locations. In 1998, 296 viable cryopreservation samples were taken from ten locations and the program expanded to include steelhead with 25 steelhead cryopreservation sample collected from Little Sheep Creek in the Imnaha River subbasin. A total of 590 cryopreserved samples taken from the Snake River basin since 1992 are in storage at Washington State University and/or University of Idaho.

The Nez Perce Tribe has and proposes to continue to enlist the assistance of: Dr. Joseph G. Cloud, a professor of Zoology in the Department of Biological Sciences at the University of Idaho, Dr. Gary Thorgaard and Paul Wheeler in the Thorgaard Lab at Washington State University, and Dr. Madison Powell, a geneticist at the Fish Genetics Lab and Hagerman Fish Culture Experiment Station with the University of Idaho. These subcontractors are experts in the field of cryopreservation of salmonid sperm and/or fish genetics. They also will advise this project on technical/research issues relative to genetics and reproductive physiology.

Selected relevant publications from the above mentioned researchers are:

Thorgaard, G.H., P.A. Wheeler and J.G. Cloud. 1998. Status and potential value of sperm banking for Snake River salmon. Proceedings of the Columbia River Anadromous Salmonid Rehabilitation and Passage Symposium (E.L. Brannon and W.C. Kinsel, editors) pp 51-56.

Nilsson, E.E. and J.G. Cloud. 1993. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres. *Aquat. Living Res.* 6:77-80.

Thorgaard, G.H. and J.G. Cloud. 1993. Reconstitution of genetic strains of salmonids using biotechnical approaches. In "Genetic Conservation of Salmonid Fishes". (J.G. Cloud and G.H. Thorgaard, editors) Plenum Press, New York, NY.

Cloud, J.G., W.H. Miller and M.J. Levanduski. 1990. Cryopreservation of salmon sperm as a means to transfer genes from wild fish to hatchery populations: A field evaluation. *Progressive Fish Culturist.* 52:51-53.

Cloud, J.G. and C.Osborne. 1997. Cryopreservation of Salmonid Fishes. University of Idaho. 44 pages.

Project description. Redd counts will be conducted to determine spawning timing and where in a stream fish are located. Post-spawned male salmonids are dip-netted from streams or collected in hatcheries. The fish will be anaesthetized. Milt or semen will be collected into a Whirl-Pak7 bag, aerated and placed in a cooler on ice. Biological information will be taken on the fish. Fin tissue samples will be taken from every male salmonid from which milt is collected, and genetic analysis will be conducted to depict the fish's genotype. The cooled semen samples are air transported to the universities for cryopreservation.

The cryopreservation process involves mixing the semen with three parts freezing solution to protect the cells during dehydration by inhibiting ice formation. The semen solution is vacuumed into straws and cooled at a rate of  $-20$  to  $-30$  °C per minute, then placed in liquid nitrogen at  $-196$  °C. The straws are stored in large liquid nitrogen tanks at the University of Idaho and Washington State University.

The use of molecular genetics for identifying what the cryopreserved inventory represents is summed up in the review by Ferguson (1995) stating that rehabilitation aquaculture should maintain as much as possible of all the genetic variation within the wild stock to maximize the probability of success. Moreover, Allendorf and Ryman (1987) state that these genetic principles should be applied during both the founding and propagation of the population. If that is so, then the identification of what the cryopreserved sperm represents is of principal importance. Without this knowledge, not only are crosses made in ignorance but the genetic diversity and portion there of in the cryopreserved archive is unknown (Powell personal communication 1998).

The Listed Salmonid Stocks Gamete Preservation project recognizes the need for new methodologies to be developed in order to cryopreserve and store fish eggs and embryos. We propose to support Dr. J.G. Cloud, University of Idaho Department of Biological Science in his investigations to cryopreserve chinook salmon eggs and/or embryos. The following is a summary of his proposed experiment (Cloud 1998):

Fish eggs are very large and compartmentalized (Hagedorn et al. 1998). Like organ systems, vitrification is the most probable method for successful cryopreservation of fish embryos (Rall and Fahy 1985; Fahy 1986). Since large amounts of cryoprotectant materials need to be incorporated into the embryo for vitrification to be successful, the objective of this proposed experiment is to identify the conditions that promote cryoprotectant uptake with minimal embryonic loss (Suzuli et al. 1995). Based on data from zebrafish (Zhang and Rawson 1995; Hagedorn et al. 1997), the proposed, initial series of studies will use chinook embryos that have completed epiboly. This study is designed as a  $4 \times 4 \times 5$  factorial; the aim of the study is to determine the relationship among the concentration of the cryoprotectant, ambient pressure and temperature. The factors to be examined are (1) concentration of cryoprotectant (0, 2,4, or 6 M of dimethylsulfoxide/DMSO), (2) ambient pressure (1, 100, 300, or 900 atms of hydrostatic pressure) and (3) temperature (10, 0, -10, -20, or  $-30$  °C). In all cases, the embryos will be reared at  $10$  °C through epiboly. The embryos will be exposed to the various treatment regimes for four hours. The cryoprotectant will be slowly removed and the embryos will be returned to the initial incubation conditions. The endpoint in this experiment will be survival to the eyeup (retinal pigmentation) stage.

#### References:

Allendorf, F. W. and N. Ryman. 1987. Genetic Management of Hatchery Stocks. In "Population Genetics and Fishery Management". University of Washington Press, Seattle, Washington.

Fahy, G.M. 1986. Vitrification: A new approach to organ cryopreservation. *Progressive Clinical Biological Research*. 224:305-335.

Ferguson, F. 1995. The role of molecular genetic markers in the management of cultured fishes. In *Molecular Genetics in Fisheries* (Carvalho, G.R. and T. L. Pitcher, editors). Chapman and Hall, London. pp 81-103.

Hagedorn, M., F.W.Kleinhans, D.E. Wildt, and W.F. Rall. 1997. Chill sensitivity and cryoprotectant permeability in dechorionated zebrafish embryos, *Brachydanio rerio*. *Cryobiology*. 34:251-263.

Hagedorn, M., F.W. Kleinhans, D. Artemov and U. Pilatus. 1998. Characterization of a major permeability barrier in the zebrafish embryo. *Biol. Reprod.* 59:1240-1250.

Rall, W.F. and G.M. Fahy. 1985. Ice-free cryopreservation of mouse embryos at  $-196^{\circ}\text{C}$  by vitrification. *Nature*. 313:573-573-575.

Suzuki, T., H. Kimada, R.Takai, K. Arii and T.T. Kozima. 1995. Relation between toxicity of cryoprotectant DMSO and its concentration in several fish embryos. *Fish Sci.* 61:193-197.

Zhang, T., and D.M. Rawson. 1995. Studies on chilling sensitivities of zebrafish (*Brachydanio rerio*) embryos. *Cryobiology*. 32:239-246.

If the Listed Salmonid Stocks Gamete Preservation Project was not funded by BPA in 2000, no further gamete collection would occur and no research would be conducted on the cryopreservation by vitrification of female gametes. The cryopreserved inventory does not hold a sufficient quantity of unique genetic material to sustain existing stocks.

#### **b. Rationale and significance to Regional Programs**

The rationale for preserving gametes of listed salmonid stocks is to serve as insurance for ongoing conservation projects. The rationale for genetically testing archived semen is to determine what the genotypes of the cryopreserved samples so the technology is used to maximize available genetic diversity within a population, minimize drift by increasing the effective population size and maintaining "population identity" (Powell personal communication 1998).

The Lower Snake River Compensation Plan hatchery evaluations program, funded through the U.S. Fish and Wildlife Service, has provided a valuable although limited amount of support for this effort since 1992. The Listed Stock Gamete Preservation project has previously received a relatively high project ranking through the Columbia Basin Fish and Wildlife Authority. The project was funded by BPA for the first time in FY 1997.

Several Northwest Power Planning Council (NPPC) program measures in the Columbia River Basin Fish and Wildlife Program (FWP) direct the implementation of the Listed Salmonid Stocks Gamete Preservation Project:

FWP measure 7.4E states Acryopreservation (preservation of fish gametes by freezing) has the potential of allowing≡banking≡ of genetic stocks for future use, especially when the population is severely depleted and its habitat has been damaged or destroyed.≡

FWP measure 7.4E.2 directs Federal and State agencies to AFund needed research and demonstrations of cryopreservation identified in the coordinated habitat and production process.≡

FWP measure 7.4D addresses captive brood stocks. ACaptive brood stock programs have the potential to rapidly increase adult fish numbers, while retaining genetic diversity of severely depleted wild or naturally spawning stocks of salmon.≡

FWP measure 7.4D.2 directs National Marine Fisheries service and Bonneville to AFund captive brood stock demonstration projects funded under the coordinated habitat and production process.

FWP measure 7.2D.2 ..... AAlso fund tests of new techniques at Columbia River basin artificial propagation facilities.≡

The National Marine Fisheries Service=s Salmon Recovery Plan states that ACaptive broodstock and supplementation programs should be initiated and/or continued for populations identified as being at imminent risk of extinction, facing severe inbreeding depression, or facing demographic risks.≡ The plan further states that Athe conservation of local populations or stocks of Pacific salmon and the preservation of their genetic resources is an important goal.≡

**c. Relationships to other projects**

The Grande Ronde captive broodstock annual operating plan for chinook salmon populations recognizes the importance of and contains guidelines for the use of cryopreserved semen to maintain genetic diversity of the propagated populations. The first use of cryopreserved semen was in September of 1998 at Bonneville Fish Hatchery to promote genetic diversity in the spawning matrix used for the Grande Ronde basin captive brood chinook salmon. The inventory of different brood year's cryopreserved semen allowed the matrix to incorporate different brood year parents for each fertilization.

Collection of male gametes from Sawtooth Hatchery, McCall Hatchery, Lookingglass Fish Hatchery (LSRCP) and Rapid River Hatchery, could be used in future low adult return years when there are not enough males to females to promote genetic diversity and to add to the genetic variability in the germ plasm repository.

The Johnson Creek Artificial Propagation Enhancement Project will use cryopreserved Johnson Creek male gametes to promote genetic diversity.

The intracytoplasmic sperm injection project (ICSI) is research into genetic retrieval from single sperm applicable to the gamete preservation project as it makes non-motile or dead sperm potentially useful for egg fertilization. This means that a low motility counts or sperm from dead salmon carcasses may be worth cryopreserving for future genetic diversity in fertilizing eggs. This could change our sampling protocols.

**d. Project history (for ongoing projects)**

The Listed Salmonid Stocks Gamete Preservation project was initiated in cooperation with the Lower Snake River Compensation Plan Tribal hatchery evaluations project in 1992 and continued through 1996 with a total of 80 samples being collected and cryopreserved from four river subbasins in the Snake River basin. The project was funded for \$110,500 by BPA (Listed Stock Chinook Salmon Gamete Preservation Project #9703800) in 1997 and the program collected 198 viable cryopreservation samples from nine locations. The project was funded for \$140,000 by BPA in 1998; a project leader was hired and the program expanded. The program collected 321 viable steelhead and chinook salmon cryopreservation samples from eleven locations. The project lent its expertise to the Grande Ronde spring chinook salmon captive broodstock project by cryopreserving male gametes from 101 salmon and thawing semen for fertilization. A total of 590 cryopreserved samples taken from the Snake River basin since 1992 are in storage at Washington State University and/or University of Idaho. A 1997 annual report was written and submitted to BPA in June of 1998.

Genetic analysis started in 1999 and will continue into 2000. This analysis has application for captive broodstock management and is necessary to characterize the genotypes of our cryopreserved inventory. Currently, the University of Idaho's Center for Salmonid and Freshwater Species at Risk has used nuclear and mitochondrial DNA polymorphisms to genetically characterize captive propagated chinook salmon from the East Fork of the Salmon River, West Fork of the Yankee Fork of the Salmon River, Salmon and Lemhi rivers. This technology is used on both live adults and cryopreserved sperm to construct a dissimilarity matrix for breeding. This matrix pairs genetically dissimilar individuals within a population to reduce the occurrence of inbreeding and problems associated with it. The matrix also evens the contributions of each genetically distinct individual and eliminates genetic drift while maximizing genetic diversity in the F1 generation. Thus, cryopreserved sperm was used in the most efficient and effective manner. The diversity in the parent population is known. Actually, two genetic types not present in the parent population was reconstituted by using cryopreserved sperm from rare individuals to effectively increase live diversity (Powell 1998).

**e. Proposal objectives**

Objective 1 - Coordinate the listed salmonid stocks gamete preservation project with management agencies and Tribes in the Snake River basin. The Tribe recognizes that salmon and steelhead are a listed species,

and strongly believes in coordination efforts to monitor, conserve, protect and recover populations at low levels of abundance and high risk of extirpation. In that regard, the Columbia River Inter-Tribal Fish Commission maintains a Section 10 permit by and through the Bureau of Indian Affairs, coordinating Tribal activities relative to listed salmon populations. An annual report is submitted to NMFS, which summarizes project activities relating to chinook salmon populations listed under the Endangered Species Act.

Objective 2 - Define cryopreservation project goals for gene banking of gametes from steelhead and chinook salmon populations at high risk of extirpation in the Snake River basin. Almost all of the listed Snake River steelhead and chinook populations are below threshold numbers of spawning adults in each population/stream and are below 10% of their historical production potential. Several chinook salmon spawning aggregates in the Middle Fork Salmon River (Big, Loon and Sulphur Creek) have experienced two to three consecutive years of cohort collapse and are at extreme risk of extirpation. Tribal and agency input for the selection of populations to be preserved (i.e. sample sizes per population, per year, and period of time to cryopreserve) has been requested. The Tribe has added steelhead to the listed species cryopreservation inventory and plans to expand the number of samples taken.

Objective 3 - Apply cryopreservation techniques to steelhead and chinook salmon conservation units at low levels of abundance and high risk of extirpation. Chinook salmon spawning times have been determined by on the ground information collected by the Nez Perce Tribe, Oregon Department of Fish and Wildlife and Idaho Department of Fish and Game. Spawning timing is further coordinated on an annual basis with redd count surveyors from each stream. Sperm samples are collected from spawned-out males and shipped to storage facilities for cryopreservation and storage processing within 12 hours. This objective is measurable by the number of samples cryopreserved each year as noted in the past accomplishments table in section 4. Genetic analysis will characterize available cryopreserved sperm from steelhead and chinook salmon in the Snake River basin to aid in captive propagation, supplementation, and recovery (Powell 1998).

Objective 4 – Cryopreservation of female genetic material

This project will support the University of Idaho's Dr. Cloud research in cryopreserving salmonid eggs and/or embryos. The objective of his study is to identify the conditions that promote cryoprotectant uptake with minimal embryonic loss. The aim of the study is to determine the relationship among the concentration of the cryoprotectant, ambient pressure and temperature. This study is the first step in a vitrification process to freeze embryos.

Objective 5 - Transfer of technology

An annual report publication summarizing all activities associated with the Listed Salmonid Stocks Gamete Preservation project will be prepared and publicized through the Bonneville Power Administration. A peer review paper detailing the preservation of salmon gametes in the Snake River basin will be presented at the Idaho Chapter of the American Fisheries Society's 2000 annual meeting.

## **f. Methods**

Objective 1 - Coordinate the listed salmonid stocks gamete preservation project with management agencies and Tribes. It is important to identify and meet with other agencies and Tribes who may help with the collection of gametes or potentially use the cryopreserved samples to accomplish this objective. Communicate with other cryogenic researchers and programs to keep current with and utilize the best technology to improve the salmonid gamete preservation program. The Columbia River Inter-Tribal Fish Commission maintains a Section 10 permit by and through the Bureau of Indian Affairs, coordinating Tribal activities relative to listed salmon populations. An annual report is submitted to NMFS, which summarizes project activities relating to chinook salmon populations listed under the Endangered Species Act.

Objective 2 - Define cryopreservation project goals for gene banking of gametes from steelhead and chinook salmon populations at high risk of extirpation in the Snake River basin. The chinook salmon spawning aggregates chosen to collect male gametes are geographically distributed throughout the Snake River basin in the Grande Ronde, Imnaha, Snake River, Salmon and Clearwater River subbasins. As many

gametes are collected as possible in the short spawning time with the limited personnel and large geographic area to cover. There has been no genetic analysis to date to measure the genetic variability of the salmonids sampled in the cryopreservation inventory. Therefore, a preliminary goal of 100 samples has been established for collection from a given spawning aggregate per year. It is recommended that a minimum population of at least 100 males and 100 females be used to sustain a hatchery population (Allendorf and Ryman 1987). If 100 samples per year can be collected over a five year time frame, this would ensure the greatest genetic diversity because chinook salmon and steelhead have approximately five years to complete a generation. Realistically, it is difficult to collect 100 individual fish from all of the streams samples simply because there are not that many adult fish returning. However, it may be possible to attain 100 samples per year in a hatchery environment. Success is not only measured in the quantity of samples collected but in the fact that we are preserving unique genetic material.

Objective 3 - Apply cryopreservation techniques to steelhead and chinook salmon conservation units at low levels of abundance and high risk of extirpation. Endangered Species Act Section 10 research permits are applied for cryopreservation purposes. Fish handling protocol training is provided to all personnel prior to collection and handling of spawned-out adult male salmon to minimize handling stress. Each team member is assigned a specific duty to improve the efficiency of sample collection. Spawned-out adult male salmon sampled are collected by hand or dip net. Pre-measured MS-222 is used to anaesthetize all adult salmon, along with a sodium bicarbonate buffering compound to buffer the acidic effect of the MS-222. Extra care is taken with semen collection to ensure the quality of preserved samples. The abdomen of the anesthetized male chinook salmon is thoroughly dried and stripped gently to reduce or eliminate contamination of the semen samples. Fish biological information (length, general condition, and external marks) is recorded following semen collection. Caudal fin tissue is collected for genetic (DNA) analysis. Scales are taken for scale pattern analysis to determine wild or hatchery origin and year class. Following sampling and data collection the anesthetized salmon are immediately returned to a slow water area and assisted until recovered. Concurrently, the semen samples are placed in two separately labeled Whirl Pak7 bags, aerated, and placed in a covered insulated cooler on wet ice on top of newspaper. Cryopreservation and storage occurs independently at the University of Idaho and Washington State University within a 12-hour period. One semen sample is stored at each university as a safeguard to protect against a catastrophic event that could destroy all germ plasm samples if they were stored at one facility.

Semen evaluation is an important component of the cryopreservation program in order to cull poor quality sperm samples prior to freezing, and to estimate the fertility of the stored sperm post-thaw. Fertility is evaluated sperm motility - percentage of motile sperm following the addition of a sperm activating solution (Mounib 1978).

There are four stages in the cooling sequence of cryopreservation of cells: 1) cooling cells to the point of ice formation; 2) the formation of ice - the goal at this stage is to have ice form near the freezing point of the extracellular solution; 3) cooling through the critical period - during this phase, there is a net movement of water out of the cells as the temperature is constantly being reduced. The rate at which the sperm is cooled is a critical factor in the success of the cryopreservation; and 4) reduction to liquid nitrogen temperature - the frozen semen is then plunged into liquid nitrogen at -196 degrees C.

Genetic evaluation is critical for threatened species, such as the steelhead and chinook salmon, especially for the use of the cryopreserved semen samples. Genetic analysis of the DNA in the frozen semen or from tissues taken from the fish sampled would help establish and maintain self-sustaining populations in captivity and in the wild. The lack of any genetic analysis would compromise the integrity of any captive-breeding program. The subcontractor doing the analysis uses mitochondrial DNA restriction fragment length polymorphisms (RFLPs), nuclear gene RFLPs of growth hormones and p53 gene introns, and microsatellite DNA using redesigned primers for PuPuPu and Om77. These three types of DNA cover the gamut of conserved and variable regions in both mitochondrial and nuclear DNA (Powell 1998).

Objective 4 – Cryopreserve female genetic material through research conducted by the University of Idaho Department of Biological Science. The proposed, initial series of studies will use chinook embryos that have completed epiboly. This study is designed as a 4 x 4 x 5 factorial; the aim of the study is to determine the relationship among the concentration of the cryoprotectant, ambient pressure and temperature. The factors to be examined are (1) concentration of cryoprotectant (0, 2,4, or 6 M of dimethylsulfoxide/DMSO), (2) ambient pressure (1, 100, 300, or 900 atms of hydrostatic pressure)) and (3) temperature (10, 0, -10, -20, or -30 ° C). In all cases, the embryos will be reared at 10 ° C through epiboly. The embryos will be exposed to the various treatment regimes for four hours. The cryoprotectant will be slowly removed and the embryos will be returned to the initial incubation conditions. The endpoint in this experiment will be survival to the eyeup (retinal pigmentation) stage.

Objective 5 – Transfer of technology occurs through coordination with Idaho Department of Fish and Game, Oregon Department of Fish and Wildlife, National Marine Fisheries Service, University of Idaho, Washington State University, the San Diego Zoological Society's Center for Reproduction of Endangered Species and additional agencies and institutions as necessary. Communication is crucial to management of listed species, especially Pacific salmonids since anadromy covers a variety of habitats, states and political jurisdictions.

The following factors may limit success of the listed salmonid stocks gamete preservation. Other agencies and Tribes may have different management perspectives with respect to the use of salmonid germ plasm. This could negatively affect this program in some critically related projects. Disagreement exists in the identification of salmonid conservation units (populations) and thus the sampling of certain streams may not be unanimously embraced. The lack of any or inadequate genetic analysis would compromise the integrity of any captive breeding program and the use of cryopreserved sperm. Equipment failure could jeopardize the quality of the cryopreserved samples.

**g. Facilities and equipment**

The McCall Field Office is currently being utilized for this project. This office provides adequate administrative space, storage and parking. The Enterprise, Oregon, Field Office is also used as a base of operation for Oregon cryopreservation sample collection. No special field equipment is required for the collection of semen samples. All specialized laboratory equipment (liquid nitrogen tanks) required for the actual cryopreservation process and research is available at Washington State University and the University of Idaho. The genetic analysis is conducted in two state-of-the-art laboratories and two automated DNA sequencers/fragment analyzers (Powell 1998).

**h. Budget**

The following proposed budget for the year 2000 is based on a calendar year.

Salaries: Consists of a project leader (\$38,000), a program leader (\$5,000), a research coordinator (\$2,000), an office manager (\$4,500), 2 technicians (\$5,000), administrative support (\$3,800), and a contract administrator (\$2,500). These salaries total **\$61,000**.

Fringe Benefits: Calculated by 27% of all permanent employees, which equals approximately **\$ 16,540**.

Training: There is **\$1,000** to be spending in training to keep current with the latest cryogenic and genetic technologies.

Travel: Travel costs include air transport of collected gametes (\$7,000), administrative support trips to Portland and Seattle as needed (\$2,000), field and city per diem rates (\$4,000). All of these above travel expenses add up to **\$13,000**.

Vehicles: Vehicle lease for 1 GSA vehicles (\$3,600), mileage which GSA charges (\$2,600), and a vehicle repair and maintenance fee (\$800) total **\$7,000.**

Office Services: Office supplies and postage (\$360), telephone services (\$600), long distance telephone charges (\$1800), internet service (\$120), photocopier rental (\$360), office rental (7,200), utilities (\$360), plus other charges such as newspaper, FedEx charges (\$200) add up to **\$11,000.**

Field Supplies: Miscellaneous supplies (\$4,000) and other provisions (\$3,000) add up to **\$7,000.**

Equipment Lease: **\$1,000** will be spent on computer equipment

Direct Costs equal **\$115,000.**

Indirect Costs equal **\$26,582** which is 23% of direct costs.

Subcontract Services: The subcontractors are University of Idaho and Washington State University (\$5,000 each), Fish Genetics Laboratories (\$7,000) plus support for University of Idaho's vitrification of female gametes research as a graduate student study (\$22,000) for a total of **\$37,000.**

The **total BPA FY2000 budget request equals \$185,122.**

Cost sharing opportunities come from the LSRCP as follows:

Salaries/Fringe Benefits: Consists of two biologists (\$2,500) and two technicians (\$1,500). These salaries total **\$9,550.**

Travel: Travel costs include field per diem rates (\$500). All of these above travel expenses add up to **\$1,110.**

Vehicles: Vehicle lease and mileage for vehicles (\$1,500) total **\$1,500.**

Direct Costs equal **\$12,660.**

Indirect Costs equal **\$2,760** which is 23% of direct costs.

The **total cost sharing amount equals \$15,420.**

The total project costs (including BPA and cost sharing opportunities) equals **\$199,944.**

## **Section 9. Key personnel**

Robyn Armstrong is the Listed Salmonid Stocks Gamete Preservation Project Leader. Ms. Armstrong has attended cryopreservation of salmonid sperm workshops and has cryopreserved semen on-site as well as in a laboratory scenario. Her knowledge of the Salmon River basin and background of 10 years working with salmonids helps her coordinate field collections. This position fills 1 FTE.

Education: Bachelor of Science, 1986 University of New Hampshire  
Major: in Pre-veterinary medicine/Animal Science

Professional Experience:

1998-present Project Leader, Nez Perce Department of Fisheries Resources Management  
Responsible for the Listed Salmonid Stocks Gamete Preservation project.

1995-1998 Fisheries Biologist, Payette National Forest, McCall, Idaho  
Supervised field data collection, wrote NEPA, reports and biological assessments.

1994 Fish & Wildlife Biologist, US Fish & Wildlife Service, Boise, Idaho  
Regulated Forest activities, conducted bull trout status review in Jarbidge basin.

1992-1994 Fisheries Biologist, Rocky Mountain Experiment Station, Boise, Idaho  
Responsible for the Desired Future Conditions Project and managed database.

1991-1992 Fisheries Technician, Idaho Department of Fish and Game, Heise, Idaho  
Surveyed and mapped the South Fork Snake River for in-stream flow study.

1991 Hydrologic Technician, Bridger-Teton National Forest, Pinedale, Wyoming  
Gathered limnological and biological data for wilderness air quality monitoring.

1989-1991 Fisheries Technician, New Hampshire Department of Fish and Game, Concord, New Hampshire

Conducted creel census, gathered data, and worked on Atlantic salmon program.

1989 Fisheries Technician, Alaska Department of Fish and Game, Valdez, Alaska

Conducted sport fish creel census and initiated bottomfish research after oil spill.

Paul A. Kucera, Director of Biological Services, Nez Perce Department of Fisheries Resources Management is the program leader for the Listed Salmonid Stocks Gamete Preservation Project. Mr. Kucera has 23 years experience as a professional fisheries biologist in research, management and administration and is a Certified Fisheries Scientist with AFS. He has authored or co-authored seven peer-reviewed fisheries journal publications and over 40 project reports. Responsible for technical program direction and administration of the Fisheries Research Division. This position fills 0.1 FTE.

Education: Bachelor of Science, 1975 Utah State University  
Major: Fisheries Management  
Graduate Studies 1984-1987 University of Idaho  
Major: Fisheries Management

Jay Hesse is the Research Coordinator, Nez Perce Department of Fisheries Resources, which supervises the project leader of the Listed Salmonid Stocks Gamete Preservation Project. Mr. Hesse has five years professional experience as a Fisheries Research Biologist and as the Research Coordinator. Responsible for technical direction and supervision of all research division projects, research coordination, and tribal fisheries research representation at federal and state meetings. This position fills 0.05 FTE.

Education: Bachelor of Science, 1992 Michigan State University  
Major: Fisheries and Wildlife  
Masters of Science, 1994 Michigan State University  
Major: Fisheries

Glenda Claire is a Fisheries Production Assistant with the Nez Perce Tribe Department of Fisheries Resources Management. Ms. Claire has attended cryopreservation of salmonid sperm workshops and has cryopreserved semen in a laboratory environment. Ms. Claire has three years experience as a production assistant with the Nez Perce Tribe and with the cryopreservation project. This position fills 0.2 FTE and is LSRCP funded.

Education: Bachelor of Science, 1987 Oregon State University  
Major: Microbiology

Michael Blenden is the Assistant Project Leader for the LSRCP hatchery evaluations project for the Nez Perce Tribe Department of Fisheries Resources Management. Mr. Blenden has seven years professional experience as a Fisheries Research Biologist with the Nez Perce Tribe. This position fills 0.05 FTE (2 weeks) and is LSRCP funded.

Education: Bachelor of Science, 1990 University of Idaho  
Major: Fisheries Management

## **Section 10. Information/technology transfer**

An annual report following scientific publication guidelines is distributed through the BPA publications system. A presentation to the Idaho Chapter of the American Fisheries Society is planned for the 1999 or 2000 annual meeting.

## **Congratulations!**