CRYOPRESERVATION OF KOOTENAI RIVER WHITE STURGEON (*Acipenser transmontanus*) GAMETES 2009 ANNUAL REPORT
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INTRODUCTION AND OVERVIEW

The following report provides an update to the Kootenai Tribe of Idaho (KTOI) on sperm cryopreservation activities for Kootenai River white sturgeon (*Acipenser transmontanus*) in 2009.

Kootenai River sturgeon are geographically isolated and genetically distinct from other Columbia River Basin white sturgeon populations (Setter and Brannon, 1990). Human activity has significantly altered the natural flows of the Kootenai River which appears to have effected the reproduction and juvenile rearing habitat of these sturgeon residing in Kootenai River and Kootenai Lake. Since completion of Libby Dam by the U.S. Army Corp of Engineers in 1972, average spring peak flows of the Kootenai River have been reduced by more than 50 percent, and winter flows have increased by approximately 300 percent. High spring flows required by white sturgeon for reproduction now occur only rarely during the spawning season, and no natural recruitment has occurred since the completion of Libby Dam (Anders et al., 2002). Consequently, Kootenai River white sturgeon were given endangered species status in Idaho and Montana in September of 1994.

An international multi-agency Kootenai River White Sturgeon Recovery Team (KRWSRT) was formed in accordance with the Endangered Species Act to develop and implement a recovery plan. Primary recovery measures are to (1) augment Libby Dam spring flows to improve spawning and juvenile rearing habitat and (2) implement conservation aquaculture and breeding plan (Kincaid, 1993) to prevent extinction (Duke et al., 1999). Conservation aquaculture is currently being used as an interim measure to prevent extinction and maintain genetic diversity (Ireland et al., 2002) until habitat restoration can restore natural recruitment.

Hatchery breeding of wild Kootenai River white sturgeon was initiated in 1990 to examine gamete viability and assess exposure to contaminants (Apperson and Anders, 1991) and was further expanded as the feasibility of producing significant numbers of juvenile fish was established (Ireland et al., 2002). Initial objectives for hatchery supplementation were to produce four to twelve separate families per year and four to ten adults per family that survive to breeding age (Kincaid, 1993). An original stocking goal of 1000 fish per family at age 15-24 months, based on expected survival rates was established (Kincaid, 1993).

Under the current breeding program, wild females are captured in early spring and brought into the hatchery where egg maturation is monitored. As wild females in captivity sexually mature, sperm is collected on the river by hatchery staff angling for

wild males. Since an inherit problem with this approach is the synchronization of male and female gamete collection to meet hatchery production goals, the development of a program to cryopreserve and store milt was initiated to insure sperm availability when captive wild females spawn. In addition to synchronizing male and female gamete availability, cryopreservation of milt is a cost-effective method to preserve paternal genetic diversity without risk of disease.

The length of time required for sexual maturation of Kootenai River white sturgeon is relatively long and presents an additional challenge for population recovery. In the Kootenai watershed, females appear to mature at age 22 and males at age 16 (Paragamian et al., 1997), with females reported to spawn once every two to eleven years (Conte et al., 1988; PSMFC, 1992). Empirical evidence suggests Kootenai River female white sturgeon have spawning periodicities of over 5 years (Paragamian et al., 2000). With virtually no natural recruitment since 1974, the Kootenai River white sturgeon population is at risk of senescence, and it is uncertain how long remaining adults will be fertile. Cryopreserved milt from remaining wild Kootenai River males would provide the paternal genetic component and could be utilized in reconstituting localized adaptation should extinction occur. Additionally this resource can serve as a long-term control to test for selective changes such as domestication which may occur in a conservation aquaculture based recovery strategy.

Here we describe Kootenai River white sturgeon milt cryopreservation efforts in our laboratory to date, and discuss potential relevance to on going recovery strategies. We present our current inventory of cryopreserved Kootenai River white sturgeon sperm samples and preliminary experimental results of six different cryoprotectant solutions on post-thaw motility, and results of three fertility trials with cryopreserved semen conducted at the KTOI Tribal Sturgeon Hatchery in June of 2009.

MATERIALS AND METHODS

Sperm collection

Kootenai River white sturgeon sperm samples were collected by Kootenai Tribal Hatchery and Idaho Department of Fish and Game personnel. Milt was obtained from wild males by using a syringe with attached polyethylene tubing inserted into the urogenital opening. Milt was stored at approximately 4 ^{o}C and gassed with oxygen for transport to the University of Idaho. Sperm quality was assessed by measuring motility. Sperm were activated using an activation solution (10mM Tris, 20 mM NaCl, 2mM $CaCl_2$, pH 8.5; Jähnichen et al., 1999) and were viewed at 100 X magnification using an Olympus BH 2 compound microscope. Relative motility was determined visually by a single operator. Only milt samples containing sperm with motility greater than 50% was frozen. Sperm was frozen immediately upon arrival to the University of Idaho; sample collection to freeze times ranged from 24-96 hours.

Cryoprotectants and Cryopreservation

In 2009, separate fertility trials were conducted at the KTOI Tribal Sturgeon Hatchery. In the first trial (Experiment #1) two different cryopreservation base extenders were compared; Tris-HCl and sucrose (118 mM Tris-HCl, 224 mM Sucrose, Tsvetkova et al.,1996), or a Tris-sucrose-KCl (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, 82 mOsmol/kg pH 8.0, Urbanyi et al., 2000). The Tris-HCl and Tris, sucrose, KCl base extenders were used to test six different freezing solution combinations:

1.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk and 15% dimethyl sulfoxide (DMSO)
2.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk and 10% DMSO
3.) 118 mM Tris-HCl, 224 mM sucrose, and 20% egg yolk with 10% MeOH
4.) 118 mM Tris-HCl, 224 mM sucrose, and 10% MeOH
5.) 30 mM Tris, 23.4 mM sucrose, and 0.25 mM KCl with 10% MeOH
6.) 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl with 20% egg yolk and 10% MeOH.

Two males (591D and 0F64) collected on May 27th and frozen on May 28th 2009 were used in Experiment # 1 along with fresh control milt from a single male. All crosses were with eggs from a single female.

A second fertility trial (Experiment #2) was conducted using fresh milt (sperm not cryopreserved) from a single male and combined with freezing solutions of:

1.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk and 15% DMSO
2.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk and 10% DMSO
3.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk and 10% MeOH
4.) 30 mM Tris, 23.4 mM sucrose, and 0.25mM KCl with10% MeOH
5.) 118 mM Tris-HCl, 224 mM sucrose, with 20% egg yolk
6.) 30 mM Tris, 23.4 mM sucrose, and 0.25mM KCl.

Experiment #3 was designed to examine the effect of cooling rate (10°C/min vs. 4°C/min) equilibration time (0 vs. 3 minutes) and ratio of milt to sperm activation solution (v: v; 1:20, 1:40, or 1:80) on fertility using sperm cryopreserved with the 30 mM Tris, 23.4 mM sucrose, and 0.25mM KCl with 10% MeOH solution in previous years (2005, 2006, and 2008).

The final trial (Experiment #4) examined cooling rates (10°C/min vs. 4°C/min) and varying MeOH concentrations (5%, 10%, or 15%) using sperm from two males from 2009 (FD00 and B4FD) cryopreserved with 30 mM Tris, 23.4 mM sucrose, and 0.25 mM KCl base extender. Both males were crossed with a single female for each treatment.

In all experiments, milt mixed with freezing solutions were drawn into 0.5mL French straws (Edwards Agri-Sales Inc., Baraboo, WI) and sealed with a sealant powder with either a 0 or 3 minute equilibrium period prior to freezing. Milt/freezing solution mixtures were frozen with liquid nitrogen vapor. Two liquid nitrogen (LN_2) vapor-freezing rates were examined by utilizing a freezing rack which suspended straws 3 cm (10°C/min) or 4 cm (4°C/min) above LN_2. Straws to be frozen were loaded into 10 mm goblets (5 straws/goblet), with two goblets being held by a single 10 cm aluminum cane. Frozen

samples were transferred directly into liquid nitrogen for long-term storage after being exposed to LN_2 vapor for 10 minutes.

Fertility Trials

Fertility trials were conducted at the Kootenai Tribal Sturgeon Hatchery in June of 2009. Eggs from a single female was used in each of the four trials. For each treatment, a single 0.5 mL straw was thawed in a 40^{o}C (104^{o} F) water bath for 10 seconds; contents of each of the thawed straws (milt diluted 1:1 which freezing solution) were emptied into a beaker containing 5 mL, 10 mL or 40 mL sperm activation solution for sperm dilutions of 1:20, 1:40, or 1:80. The resultant mixture (containing activated sperm) was then added to a small beaker containing approximately 100 eggs (to be fertilized) and swirled 2 minutes. These treated eggs were washed once with hatchery water and then transferred directly into a 15 x 100 mm petri dish submerged in approximately 3 cm of water in a KTOI flow through rearing trough for incubation (no egg de-adhesion process was used). Fertilization success was determined at 12 hours post-fertilization as a viable embryo in late cleavage stage of development with numerous micromeres present in the animal hemisphere; percent fertilization was determined from the number of developing embryos divided by the total number of embryos incubated (ranged between 100 and 130 embryos) times 100.

Statistical Analysis

In Experiment #2, base extender toxicity, cryoprotectant toxicity, and sperm activation dilution were tested using an analysis of variance (ANOVA) with SAS statistical software (SAS Version 9.2). Independent effects analyzed with a fixed model in Experiment # 2 were base extender and cryoprotectant. A mixed model ANOVA with random male effect was used to analyze effect of sperm activation dilution in Experiment # 2. Least square means were compared using a Tukey-Kramer multiple comparison test. No statistical analysis was conducted in Experiments 1 and 3 due to small sample size and overall lack of fertilization.

RESULTS

In 2009, milt from an additional 11 males were collected and frozen at the University of Idaho. Total number of Kootenai River white sturgeon semen samples cryopreserved and held at the University of Idaho for the KTOI is currently 83 (Table 1).

Table 1. Inventory of cryopreserved Kootenai River white sturgeon sperm samples held at the University of Idaho as of September 2009. Samples are identified by the last for digits of their pit tag number.

2004		***2005***		***2006***	
2972	7B37	261A	606E	334A	2A46
106C	7744	0715	585A	356A	165A
261A	117A	472A	317A	561A	7259
OE6A	6276	237A	7848	824	6B60
207D	2F5A	7D20	B7D8	626B	146A
5351	3672	1C67	580B	3E40	651A
5E5C	6532	776E		227A	155A
080F	6453			3E51	
2971	7870				

2007		***2008***		***2009***	
460A	513A	20A8	3414	4E2E	004B
163A	451E	CF34	4D86	59D1	FE32
6D0E	2A1B	5A76	271A	A00B	4134
334C	116A	4BF0	11E0	37F5	FD00
625A	4573	OE47	2BDE	FD8A	B4FD
3D5A		1899	13DB	0F64	
		F8C9	4D7D		
		2E74			

Fertility trials at the Kootenai Tribal Sturgeon Hatchery

Experiment #1

Experiment #1 examined the effect of two different cryopreservation base extenders; Tris-HCl and sucrose (118 mM Tris-HCl, 224 mM Sucrose, Tsvetkova et al., 1996) and Tris-sucrose-KCl (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, Urbanyi et al., 2000) in six different freezing solutions. No development was observed at 12 hours post-fertilization in eggs in which cryopreserved milt was added; however, 89.5% of eggs mixed with fresh control milt were fertilized (Table 2). All cryopreserved milt in experiment #1 incorporated a 3 minute equilibration time and a cooling rate of 4 °C/minute.

Table 2. Experiment #1fertility results.

Freezing Solution	*Base Extender*	*cryoprotectant*	*Egg yolk*	*Cooling rate*	*Equilibration time*	*Fertilization rate (%)*
1	118 mM Tris-HCl, 224 mM Sucrose	15% DMSO	20 %	4° C/min	3 min	0
2		10% DMSO	20%	4° C/min	3 min	0
3		10% MeOH	20%	4° C/min	3 min	0
4		10% MeOH	0%	4° C/min	3 min	0
5	30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl	10% MeOH	0%	4° C/min	3 min	0
6		10% MeOH	20%	4° C/min	3 min	0
	Fresh Milt control					89.5

Experiment #2

Experiment # 2 compared the toxicity of freezing solutions used in Experiment #1. Fertilization rates greater than 90% were recorded at 12 hours post-fertilization with fresh milt in all crosses except the 118 mM Tris-HCl, 224 mM sucrose with 15% DMSO, which had a 68.9% fertilization rate (Table 3). The amount of cryoprotectant was found to have a significant effect on fertilization; the fertilization rate was significantly lower when 15% DMSO was used as the cryoprotectant. There was no detectable effect of the base freezing solution; 118mM Tris-HCl, 224 mM sucrose and 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl base extenders were not statistically different (Table 4).

Table 3. Freezing solution toxicity on fresh milt fertilizing ability. Fresh milt exposed to 3 minute equilibration time prior to fertilizing eggs in all crosses.

Freezing Solution	*Base Extender*	*cryoprotectant*	*Egg yolk*	*Fertilization rate (%)*
1	118 mM Tris-HCl, 224 mM Sucrose	15% DMSO	20 %	68.9
2		10% DMSO	20%	97.2
3		10% MeOH	20%	95.1
4		0% MeOH	0%	91.0
5	30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl	10% MeOH	0%	98.0
6		0% MeOH	20%	95.6
	Fresh Milt control			96.2

Table 4. Two-way ANOVA analysis of base extender and cryoprotectant effect on fertility.

Source of variation	*DF*	*F-value*	*P-value*
Base extender	1	19.46	0.14
Cryoprotectant	**3**	235.76	**< 0.05**

Experiment #3

Experiment # 3 compared the effect of cooling rate, equilibration time, and sperm activation dilution on fertility with milt samples frozen in 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl and 10% MeOH. The proportion of eggs that were fertilized was examined 12 hours after adding milt. Fertility was observed in all eggs fertilized with milt frozen at 10°C/minute without an equilibration time. Fertility rates ranged from 39.6% (Male 317A-05) to 4.3% (Male B7D8-05). No fertilization occurred with milt frozen from male 4D7D-08 frozen in 2008 with a 3 minute equilibration time and 4°C/minute cooling rate (Table 5). The dilution rate of the milt/freezing solution and sperm activator did not have significant effect on fertilization rates (Table 6). We could not statistically determine if cooling rate and equilibration time had a significant effect since no fertilization occurred in male 4D7D; however the lack of fertilization suggests a strong adverse effect when a 4° C/min and 3 minute equilibration time is used to cryopreserve milt.

Table 5. Fertility of cryopreserved sperm relative to rates of dilution at activation.

Male ID #	*Year Frozen*	*Cooling rate*	*Equilibration time*	*Sperm Dilution*	*Fertilization rate (%)*
B7D8-05	2005	10° C/min	0 min	1:20	4.3
				1:40	6.0
				1:80	6.1
317A-05	2005	10° C/min	0 min	1:20	21.2
				1:40	38.9
				1:80	39.6
824-06	2006	10° C/min	0 min	1:20	24.8
				1:40	22.0
				1:80	21.4
4D7D-08	2008	4° C/min	3 min	1:20	0.0
				1:40	0.0
				1:80	0.0

Table 6. Mixed model ANOVA with fixed sperm activation dilution effect on milt frozen at 10° C/min with 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl and 10% MeOH with no equilibration prior to freezing. Mixed model includes random male effect.

Sperm Activation Dilution	*Mean Fertilization (%)*	*DF*	*F -value*	*P-value*
1:20	29.7			
1:40	22.3	2	0.76	0.53
1:80	22.4			

Experiment #4

In Experiment #4 cooling rates of 4 °C/minute and 10 °C/minute with varying MeOH concentrations (5%, 10%, and 15%) were examined using sperm from males FD00 and B4FD collected in 2009, cryopreserved with 30 mM Tris, 23.4 mM sucrose, and 0.25 mM KCl base extender. No fertilization was documented at 12 hours post-fertilization except in Male B4FD, with milt frozen in 15% MeOH concentration and frozen at 10 °C/minute, where a fertilization rate of 0.138 was recorded (Table 7).

Table 7. Effect of cooling rate (3 cm versus 4 cm) and varying MeOH concentrations on milt cryopreserved with 30 mM Tris, 23.4 mM sucrose base extender.

Male ID #	*Cooling rate*	*MeOH Concentration*	*Fertilization rate (%)*
FD00	4° C/min	5 %	0.0
		10%	0.0
		15%	0.0
	10° C/min	5 %	0.0
		10%	0.0
		15%	0.0
B4FD	4° C/min	5 %	0.0
		10%	0.0
		15%	0.0
	10° C/min	5 %	0.0
		10%	0.0
		15%	**13.8**

DISCUSSION

Post-thaw motility has been used widely as a measure of cryopreserved sperm quality in numerous species; however, our results are consistent with the conclusion that post-thaw motility is not an accurate predictor of fertility of cryopreserved white sturgeon sperm. For example, in previous studies we have achieved relatively high post-thaw motilities but have not been able to produce viable embryos in fertility trials (Patton et al., 2008). One reason for this discrepancy is that sturgeon sperm (unlike sperm of other fishes) have an acrosome, a modified secretory vesicle that enables the sperm to penetrate extracellular surface coats of the egg, and that the acrosome may be damaged during the freezing process even though motility remains high. An assay to assess sturgeon acrosome integrity has recently been developed. In sterlet sturgeon, a soybean trypsin inhibitor (SBTI) conjugate with AlexaFluor ® 488 and computer-aided sperm analysis (CASA) was used to detect significant differences in acrosome integrity between fresh sperm and cryopreserved sperm (Psenicka et al., 2009). Using methods described by Psenicka et al., we would like to better predict which freezing solutions will be the best candidates for use in fertility trials at the KTOI Tribal Sturgeon Hatchery. We are developing the SBTI-Alexa conjugate to assay acrosome membrane integrity of cryopreserved Kootenai River sperm; we will continue to compare our results on acrosome integrity and fertility of frozen sperm

In Experiment #2, fresh milt from a single male was exposed to the freezing solutions used in Experiment #1. Our results indicate that the components of the extenders, the presence of egg yolk or the use of MeOH and DMSO (up to 10%) did not compromise fertilization (affect the fertility of the sperm or the egg). However, DMSO at a concentration of 15% had a significant detrimental effect resulting in reduction in fertilization rates (Tables 3 and 4).

Our objective in Experiment #3 was to test whether sperm concentrations were too high during our fertility trials, possibility leading to polyspermy. Using milt dilutions of 1: 20, 1: 40 and 1: 80 (v:v), no significant effect of sperm concentration on fertilization were detected (Table 6). This is an important result; it clearly indicates that we can dilute the sperm (and as a result, the cryoprotectant) without loss of fertility. Perhaps a more important finding in Experiment #3 is that sperm frozen at the faster cooling rate (10 ° C/ minute) with no equilibration time were fertile post-thaw (Table 5).

Culture procedures to test embryonic development as a measure of fertility implemented in 2009 have resulted in the highest and most consistent fertilization rates to date. Working with single straws and small egg lots appears to minimize errors that may occur when attempting to thaw multiple straws simultaneously. This method was successfully replicated at the Kootenay Trout Hatchery (Fort Steele, British Columbia) to hatch white sturgeon embryos derived from white sturgeon milt cryopreserved with 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl base extender using 10% MeOH and a 10 °C/minute freeze rate

(unpublished data, 2009). To our knowledge, this is the first time live embryos have been produced with cryopreserved white sturgeon milt.

An important caveat in all sperm cryopreservation experiments is to consider male variation and efforts should be made to collect and freeze milt at the height of the spawning season, when milt is at its highest quality. Males collected at the beginning and end of the spawning season appear to produce milt much less tolerant to the freezing process, and subsequently result in reduced fertility rates. Results from 2009 lead us to recommend collecting and cryopreserving milt at the peak of the spawning season with 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl base extender and 10% MeOH with a 10 °C/minute freeze rate. Since the data support the conclusion that freezing solutions are not toxic, improved fertilization rates are expected using different dilution rates with higher sperm to egg ratios.

In summary, we propose the following strategies be implemented in the cryopreservation of Kootenai River white sturgeon milt to aid in population recovery:

1.) Use of 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl as milt extender to cryopreserve Kootenai River white sturgeon milt.

2.) Use of 10% MeOH as the cryoprotectant in the freezing media.

3.) A cooling rate of 10° C/minute.

4.) Using fertilization rate to assay fertility of sperm post-thaw (use of petri dishes culture of fertilized eggs and use of small egg lots of approximately 100 eggs or fewer).

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