CRYOPRESERVATION OF KOOTENAI RIVER WHITE STURGEON (*Acipenser transmontanus*) GAMETES 2008 ANNUAL REPORT

A report to the Kootenai Tribe of Idaho September 2008

Prepared by Steven J. Patton, Manasi Kanuga, and Joseph G. Cloud
Department of Biological Sciences, University of Idaho

INTRODUCTION AND OVERVIEW

The following report provides an update to the Kootenai Tribe of Idaho (KTOI) on the cryopreservation and storage of germ cells derived from the Kootenai River white sturgeon (*Acipenser transmontanus*) in 2008.

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 effecting spawning, egg incubation, juvenile rearing habitat, and reducing overall productivity of the Kootenai River and Kootenay Lake. Completion of Libby Dam by the U.S. Army Corp of Engineers in 1972 is considered to be a primary reason for the population's continued decline. Completion of Libby Dam reduced Kootenai River average spring peak flows by more than 50 percent, and winter flows were increased by approximately 300 percent. Natural high spring flows required by white sturgeon for reproduction now occur only rarely during the spawning season. Since the completion of Libby dam no natural recruitment has occurred (Anders et al., 2002). Kootenai River white sturgeon received 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 by the U.S. Fish and Wildlife Service 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 by KTOI 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 can be monitored. As wild females in captivity ripen, sperm is collected on the river by hatchery staff angling for wild males. An inherent problem with this approach is synchronizing male and female gamete collection to meet hatchery production goals; milt cryopreservation could ensure sperm is available when captive wild females ripen. In addition to synchronizing male and female gamete availability, cryopreservation could prove to be a cost-effective way to indefinitely preserve paternal genetic diversity without risk of disease until sufficient habitat restoration to restore recruitment has occurred.

Slow sexual maturation of Kootenai River white sturgeon presents an additional challenge for population recovery. In the Kootenai watershed, females have been documented 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 preserve the paternal genetic component and could be utilized in reconstituting localized adaptation should extinction occur and 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 fertility trials with cryopreserved semen conducted at the KTOI Tribal Sturgeon Hatchery in July of 2008.

MATERIALS AND METHODS

Sperm collection

Kootenai River white sturgeon sperm samples were collected by Kootenai Tribal Hatchery personnel 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. Sperm was stored at approximately 4^{o}C and gassed with oxygen for transport to the University of Idaho. Sperm quality was assessed by a sperm motility check shortly after arrival using a sperm activation solution (10mM Tris, 20 mM NaCl, 2mM $CaCl_2$, pH 8.5; Jähnichen et al., 1999).

Activated semen was viewed with 100 X magnification on an Olympus BH 2 compound microscope; relative motility was determined visually by a single operator. Sperm was frozen immediately upon arrival to the University of Idaho; sample collection to freeze times ranged from 24-96 hours.

Cryoprotectants and Cryopreservation

Six different freezing solutions were used cryopreserve milt: 1.0) Tris-sucrose-KCl (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, 82 mOsmol/kg pH 8.0; Urbanyi et al., 2000); 2.) 37.1% sucrose, 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, and 10% DMSO (Trukshin, 2000); 3.) 0.3 M dextrose, 30 mM Tris, pH 8.5 with 13% egg yolk and 10% DMSO; 4.) 125mM Tris-HCl, pH 8.0, with 12.5% yolk and 10 % DMSO (Cherepanov and Kopeika, 1999); 5.) 37.1% sucrose, 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, and 10% MeOH; and 6.) 118 mM Tris-HCl, 224 mM sucrose with 20% egg yolk and 15% DMSO (Tsvetkova et al., 1996).

Semen and freezing solutions were drawn into 0.5mL French straws (Edwards Agri-Sales Inc., Baraboo, WI) and sealed with a sealant powder with no equilibrium period prior to freezing. Semen/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 4 cm or 5 cm 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. Filled straws were loaded into goblets were transferred to the freezing rack with straws 3 cm distance above liquid nitrogen. Frozen samples were transferred directly into liquid nitrogen for long-term storage after being exposed to LN_2 vapor for 10 minutes.

Post-thaw Motility Analysis

Three straws were thawed for each male per treatment. Straws were thawed in a 40 ^{o}C water bath for 13 seconds. Contents of 0.5 mL straws were then emptied into 15 ml of sperm activation solution (10 mM Tris, 20 mM NaCl, 2 mM $CaCl_2$, pH 8.5; Jahnichen et al., 1999) for 1:61 dilution ratios. 2 µl of activated semen was transferred to a CoverWell™ perfusion chamber gasket (eight chambers, 9mm diameter, 0.5mm deep) for computer assisted sperm analysis (CASA). Motility was recorded at two time points (15 and 45 seconds post-activation) with a camera utilizing a high frame rate (97 fps) coupled with a trinocular tube on an inverted phase contrast, Zeiss Axiovert 40 microscope. Recorded images were imported as image sequences for analysis with Image J software from the National Institute of Health. A total of four motility parameters were analyzed: percent motility, point to point velocity (VCL), path curvature (LIN), and side-to-side movement of the sperm head (WOB).

Fertility Trials

Fertility trials were conducted at the Kootenai Tribal Sturgeon Hatchery in July of 2008. A single female and two males were used in the cryopreservation fertility trials. Four freezing protocols were tested based on preliminary post-thaw motility assessments conducted on a domestic sturgeon population; each protocol tested utilized an base extender solution of 37.1% sucrose, 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, with 10% MeOH or 10% DMSO as the cryoprotectant (Trukshin, 2000) at two different cooling rates (4 cm or 5cm above LN_2).

Ten 0.5 mL straws were thawed in a in 40^{o}C (104^{o} F) H_2O bath for 15 seconds; contents of thawed straws were emptied into beaker containing 250 mL sperm activation solution for a final 1:100 sperm/activator dilution. Activated semen was then added to small stainless steel bowl containing eggs to be fertilized and swirled until eggs began to adhere at approximately 3 minutes. An H_2O/aggregate was then added and gently stirred with feather for egg de-adhesion. Upon de-adhesion, fertilized eggs were placed into 2 liter McDonald style incubators for development.

RESULTS

In 2008, milt from an additional 15 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 72 (Table 1).

Post-thaw motility

The highest mean post-thaw motilities at 15 and 45 seconds were recorded with 37.1% sucrose extender with 10% DMSO, followed by Tris-HCl, sucrose with 20% egg yolk and 15% DMSO, Dextrose-Tris with 13% egg yolk and 10% DMSO, Tris-HCl with 12.5% yolk and 10 % DMSO, 37.1% sucrose with MeOH, and Tris-Sucrose-KCl with 10% MeOH (Table 2, Fig 1). A delayed activation was recorded with semen cryopreserved with 37.1% sucrose extender when MeOH was used as the cryoprotectant. Peak motility was recorded at 60 seconds post-thaw for semen cryopreserved with 37.1% sucrose extender when MeOH was the cryoprotectant (Fig. 2).

Fertility trials at the Kootenai Tribal Sturgeon Hatchery.

Based on high post-thaw motility data (Table 2, Fig 1), fertility trials at the Kootenai Tribal Sturgeon Hatchery were conducted only with semen cryopreserved with the 37.1% sucrose based extender. Attempts using milt from

two males to fertilize egg batches of 1000 were not successful. No fertilization was documented. Fresh milt controls had high fertilization rates of over 90% to neurulation.

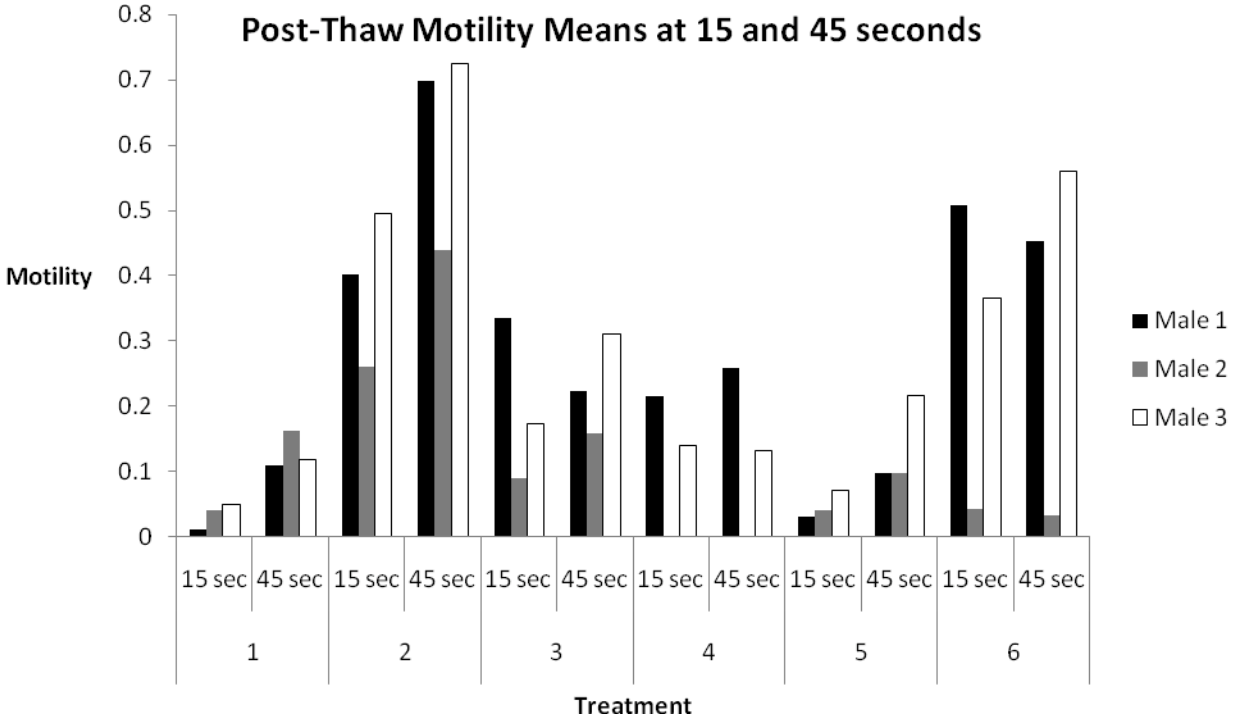
Table1. Inventory of cryopreserved Kootenai River white sturgeon sperm samples held at the University of Idaho as of September 2008. Samples are identified by the last for digits of their pit tag number.

2004		***2005***		***2006***		***2007***		***2008***	
2972	7B37	261A	606E	334A	2A46	460A	513A	20A8	3414
106C	7744	0715	585A	356A	165A	163A	451E	CF34	4D86
261A	117A	472A	317A	561A	7259	6D0EE	2A1B	5A76	271A
OE6A	6276	237A	7848	824	6B60	334C	116A	4BF0	11E0
207D	2F5A	7D20	B7D8	626B	146A	625A	4573	OE47	2BDE
5351	3672	1C67	580B	3E40	651A	3D5A		1899	13DB
5E5C	6532	776E		227A	155A			F8C9	4D7D
080F	6453			3E51				2E74	
2971	7870								

Table 2. Mean post-thaw motility 45 seconds. Treatments: 1 = Tris-Sucrose-KCl with 10% MeOH; 2 = 37.1% sucrose with 10% DMSO ; 3 = Dextrose, Tris, 13 % yolk with 10% DMSO; 4 = Tris-HCl with 12.5 % yolk and 10% DMSO; 5 = 37.1% sucrose with 10% MeOH; 6 = Tris-HCl with 20 % yolk and 15% DMSO.

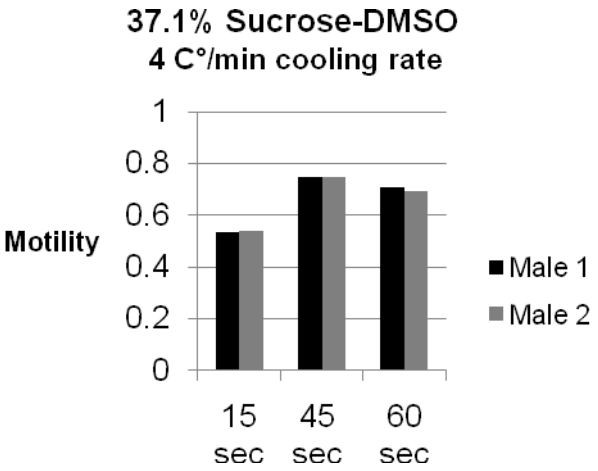
Male	***Initial Motility***	***Treatment***	***Post-Thaw Motility***
1	0.851	1	0.110
		2	0.698
		3	0.223
		4	0.258
		5	0.099
		6	0.453
2	0.734	1	0.163
		2	0.440
		3	0.159
		4	0
		5	0.098
		6	0.033
3	0.928	1	0.118
		2	0.725
		3	0.311
		4	0.132
		5	0.217
		6	0.560

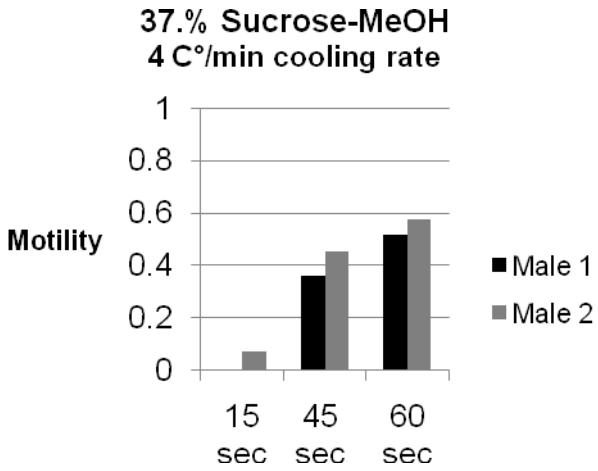
Fig.1 Mean post-thaw motilities at 15 and 45 seconds for the six freezing treatments tested.

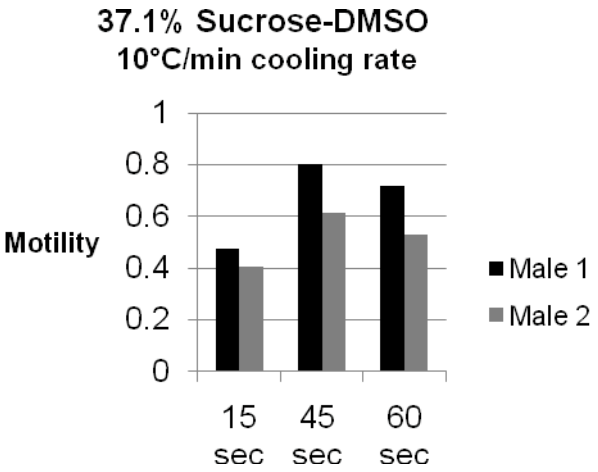


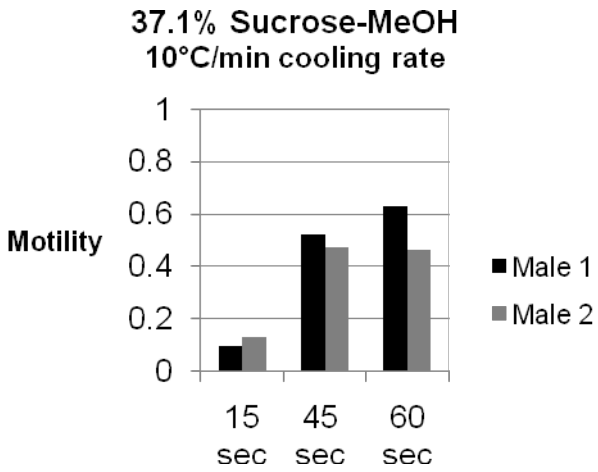
1. Tris-Sucrose-KCl with 10% MeOH
2. 37.1 % Sucrose with 10% DMSO
3. Dextrose, Tris, 13% egg yolk and 10% DMSO
4. Tris-HCl, with 12.5% yolk and 10 % DMSO
5. 37.1% sucrose with 10% MeOH
6. Tris-HCl, sucrose with 20% egg yolk and 15% DMSO

Fig 2. Mean post-thaw motility for 37.1% sucrose extender with MeOH and DMSO as cryoprotectants at 4°C/min and 10°C/min cooling rates.









PRIMORDIAL GERM CELLS

Primordial germ cells (PGCs) are a population of cells that are segregated during early development at an extragonadal site. Following gastrulation, these primordial germ cells will migrate to the developing genital ridges and develop into the gametes.

PGCs have been transferred between amphibian embryos; the protocol in these experiments was surgical removal of a ventral component of a neurula and transplantation of the excised tissue into the same position in a recipient (Blackler and Fishberg, 1961; Blackler, 1962). Although this methodology has been successful for Xenopus, utilizing this technique as a component for the cryopreservation and storage for sturgeon PGCs is problematic. It requires the removal of the jelly layer, the freezing of tissue with cells containing yolk and transplantation of the tissue post-thaw.

Using a fish species, rainbow trout, it has recently been demonstrated that testicular, germinal stem cells appear to have the same biological potential as PGCs (Okutsu et al., 2006). Injection of cells derived from enzymatically dispersed testes of rainbow trout into the coelomic cavity of hatched embryos resulted in the migration of these germinal stem cells to and the colonization of the genital ridges. The introduced germ cells participated in the development of the gonads, spermatogonial stem cells injected into female embryos developed into oocytes. This technology has been successfully applied (Okutsu et al., 2007). In fact, our laboratory at the University of Idaho is currently involved in a project utilizing this technology. We presently have successfully cryopreserved sexually immature testes from Redfish Lake sockeye salmon (as measured by intact plasma membranes of resultant cells and metabolically active mitochondria; tissues stored in liquid nitrogen for times exceeding 1 year), enzymatically digested the tissues post-thaw, and injected the resultant cells (approx 30,000/embryo) intraperitoneally into newly hatched, female, sterile rainbow trout embryos. The expectation of this project is that the surrogate rainbow trout females will produce only Redfish Lake sockeye salmon eggs derived from post-thaw testicular cells.

From our initial activities, we have made the following conclusions:

- Sturgeon neurula are difficult to manipulate (they do not survive following extensive manipulation)
- Sexually immature testes appear to be a more appropriate source of germinal stem cells

We are presently in the process of developing a protocol to cryopreserve sturgeon testes. The experimental parameters are (1) cryoprotectant, (2) cooling rate and (3) thawing rate. The endpoint for these studies will be the proportion of cells that are viable (have an intact plasma membrane). At the same time, we will

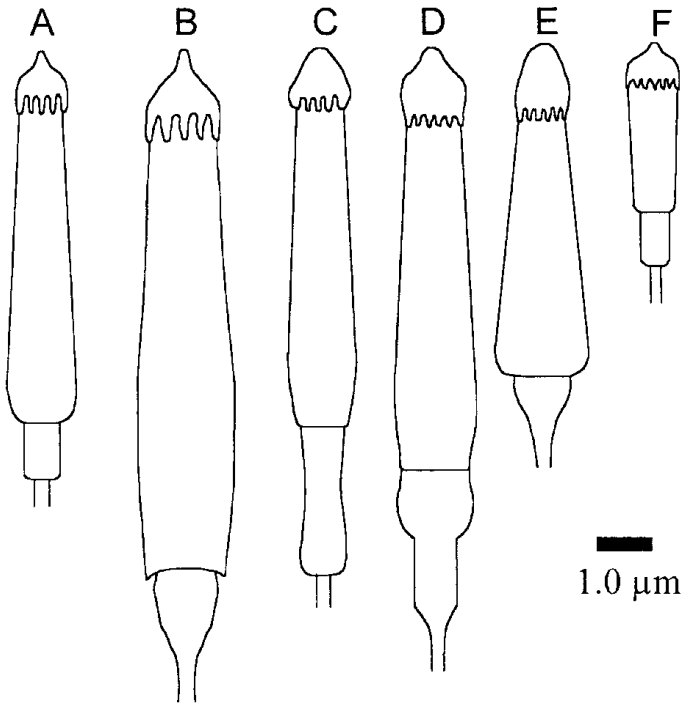
be working on the digestion of the testes and the injection of the resulting cells into early embryos.

DISCUSSION

Results from our laboratory in 2008 indicate extender solutions have a significant effect on post-thaw motility. We have demonstrated a 37.1% sucrose based freezing solution can produce high post-thaw motilities in white sturgeon; however we were unable to achieve any egg fertilization with sperm frozen with a 37.1% sucrose based extender when either DMSO or MeOH was used as the cryoprotectant. The inability to achieve any fertilization in fertility trials conducted at the Kootenai Tribal Sturgeon Hatchery with cryopreserved milt is unclear. In our control fertilizations with fresh milt, fertility rates of greater than 90% were achieved. Motility rates are generally regarded as a strong indicator of milt quality in aquaculture, but high post-thaw motilities and low or non-existent fertilization rates has been documented in other sturgeon species (Lahnsteiner et al., 2004; Horvath et al., 2005; and Linhart et al., 2006). DMSO has been widely used as a cryoprotectant for freezing semen of numerous fish species, and has been used successfully to cryopreserve semen in other sturgeon species (Tsvetkova et al., 1996; Trukshin, 2000). MeOH has also been demonstrated to be an effective cryoprotectant in fish. Horvath and Urbanyi (2000) were the first to successfully use MeOH as a cryoprotectant in a sturgeon species (*Acipenser ruthenus L*). Glowgowski et al. (2002) used a Tris-Sucrose-KCl extender with 10% MeOH to fertilize eggs and achieve hatch rates of 29.6 ± 5.0% with Siberian sturgeon (*Acipenser baeri,* Brandt). Despite the lack of success in fertility trials at the Kootenai Tribal Sturgeon Hatchery, we feel MeOH and DMSO are still well-suited for use as cryoprotectants based previous studies and our high post-thaw motilities.

Fertility trials at the Kootenai Tribal Sturgeon Hatchery were conducted only with milt frozen with 37.1% sucrose extender based on post-thaw motility analysis (Table 2, Figs 1 and 2), and the physical similarities in the cell size of white and stellate sturgeon sperm (Fig 3). Trukshin (2000) reported post-thaw motilities of 40-50% and a fertilization rate of 22% in stellate sturgeon when using a 37.1% sucrose extender with DMSO as the cryoprotectant with a cooling rate of 4°C/min. Based upon post-thaw motility rates of over 60% when semen was cryopreserved at both 4° C/min and 10° C/ min cooling rates (Fig 2), we hypothesized the freezing process that proved effective in stellate sturgeon would translate to white sturgeon given similarities in their sperm morphology. But results from the Kootenai Tribal Sturgeon Hatchery indicate high post-thaw motility is not an accurate indicator of sperm capacity to fertilize eggs.

Fig. 3. Drawings to scale of various sturgeon sperm cells. Figures taken from DiLauro et al. 2001. (A) Lake sturgeon (*Acipenser fulvescens*). (B) White sturgeon (*Acipenser transmontanus*). (C) Russian sturgeon (*Acipenser gueldenstaedti colchicus*). D) Stellate sturgeon (*Acipenser stellatus*). (E) Chinese sturgeon (*Acipenser sinensis*). (F) Atlantic sturgeon (*Acipenser oxyrhynchus*).



A reason for reduced/non-existent fertilization rates in cryopreserved sturgeon milt when post-thaw motilities are high is unclear. It seems likely the acrosome, a specific structure that enables the sperm to penetrate the egg, may be damaged during the freezing process even though motility remains high. If this assumption is correct, an assay to assess post-thaw acrosome damage would be a valuable tool in evaluating the potential fertilizing ability of cryopreserved milt.

Psenicka et al. (2008) have recently developed a fluorescent technique for visualizing the sturgeon acrosome. In sterlet sturgeon, Psenicka et al. used a soybean trypsin inhibitor (SBTI) conjugate with AlexaFluor ® 488 and computer-aided sperm analysis (CASA) to detect significant differences in acrosome staining between fresh sperm and sperm cryopreserved with DMSO or MeOH. Psenicka et al. recorded specific acrosome staining of fresh milt to be approximately 5%; semen cryopreserved with MeOH had specific acrosome staining of approximately 6%, while sperm cryopreserved with DMSO had

significantly higher acrosome staining of approximately 12%. These acrosome staining percentages correspond to recorded fresh semen motilities of 76.6 ± 4.4%, and post-thaw motilities for MeOH cryopreserved semen of 48.0± 8.8% and 39.0 ± 9.3% for semen frozen with DMSO.

In the Psenicka study, most sperm acrosomes were probably not stained with the SBTI-Alexa conjugate because the acrosome membrane was intact. Sperm with damaged acrosomes should have higher rates of staining. If this is correct, the SBTI-Alexa conjugate could be used to assay acrosome integrity of cryopreserved semen. In this manner, various freezing methods could be tested by thawing cryopreserved semen samples and recording post-thaw motilities and determining the status of acrosome integrity. Freeze methods with reasonable levels of post-thaw motility and high levels of acrosome integrity could then be chosen for fertility trials. In the future, we recommend the development and implementation of a post-thaw acrosome staining assay for white sturgeon to ascertain which freezing methods allow for the greatest likelihood of fertilization to occur. By comparing the acrosome staining of fresh milt to cryopreserved milt, a baseline measure should be attainable to quantify which freezing protocols produce post-thaw acrosome integrity that most closely resembles that of fresh milt. In this manner, various freezing methods can be tested in the laboratory to identify which methods are the best candidates to use for future motility trials.

To date, our laboratory has only been successful in producing viable embryos (to neurulation) using a Tris-sucrose-KCl based freezing solution with 10% MeOH. In 2006, fertility trials conducted at the KTOI Sturgeon Hatchery we were able to achieve fertilization rates ranging from 0.05 to 0.24 in multiple trials, the highest recorded mean fertilization rate was 0.15± 0.07. Mean post-thaw motilities of semen used in the 2006 fertility trials for the KTOI ranged from 0.043 to 0.125 (Patton et al., 2006). We recommend the continued use of the Tris-sucrose-KCl extender, along with the other extenders listed in this study. It is unclear if DMSO can be an effective cryoprotectant in white sturgeon. Results from Psenicka et al. suggest DMSO can have a deleterious effect on the acrosome of cryopreserved sturgeon milt, significantly compromising the sperm's fertilizing capacity. In sea urchins, DMSO has been found to induce the acrosome reaction (Mikami-Taki et al., 1987). It is plausible white sturgeon semen may be more prone to acrosome damage when DMSO is used as a cryoprotectant. However we were unable to produce any fertilization with MeOH as a cryoprotectant when used in conjunction with the 37.1% sucrose extender. At this point we can only conclude 37.1% sucrose is not an effective cryoprotectant extender and should no longer be used. More research is needed to determine if DMSO can be an effective cryoprotectant for white sturgeon. Additional studies are also needed to determine the optimal cooling rate for freezing white sturgeon semen.

In summary, we recommend the following strategies be followed for using cryopreservation to aid in population recovery of Kootenai River white sturgeon:

1.) The continued use of Tris-sucrose-KCl, Dextrose-Tris-13% yolk, Tris-HCl with 12.5 % yolk, and Tris-HCl with 20 % yolk extenders to cryopreserve Kootenai River white sturgeon milt. We do not recommend the continued use of 37.1% sucrose as a freezing solution based.

2.) The use of a soybean trypsin inhibitor (SBTI) conjugate with AlexaFluor ® 488 to develop an acrosome staining assay, along with computer-aided sperm analysis (CASA) to determine freezing protocols mostly likely viable post-thaw semen.

3.) The continued use of MeOH and DMSO as cryoprotectants. Sturgeon milt frozen with both MeOH and DMSO have demonstrated fertilizing capacity. It is unclear which cryoprotectant is more suitable for use with white sturgeon.

4.) The continued use of a 10° C/min and 4° C/ min cooling rate. Due to the relatively large size of white sturgeon sperm cells we recommend more experiments to determine if a faster or slower cooling rate induces less damage to cryopreserved cells.

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