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ARTICLE

Quality Assessment of Wild Atlantic Sturgeon Semen under Conditions of Short-Term Storage

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Abstract

Short-term storage trials were conducted with semen of Atlantic sturgeon *Acipenser oxyrinchus* collected from a total of nine wild males during the 2008 and 2009 spawning seasons on the Hudson River. Semen samples were kept refrigerated (4 ± 1°C) and stored in different gaseous atmospheres and storage extenders. The gaseous environments included oxygen, nitrogen, and air, while the extender treatments included modified Tsvetkova (MT), Park and Chapman (PC), and undiluted. Analyses of four response variables—percent viable sperm (viability), percent motile sperm (motility), curvilinear velocity (VCL), and ATP content—were determined on the day of arrival in the laboratory and then every other day for 7 d in 2008 and 21 d in 2009. The experiments were conducted in a split-split-plot design to examine dilution treatment, gas atmosphere, and repeated measures across days, and linear analyses of covariance models were fit to the data after appropriate transformation. Storage of sperm in an oxygen atmosphere was superior to storage in a nitrogen atmosphere for all variables. Storage of semen in 100% or 21% (air) oxygen in PC did not lead to differences among variables except for viability; viability was maintained at more than 95% through 13 d, decreasing to 88% and 94% on day 21 in 100% oxygen and air, respectively. During the last 5 d survival in an air atmosphere was better than that in 100% oxygen. Motility decreased during the 21 d of storage and the decline was greater in undiluted than in PC-extended semen, decreasing from 85% on day 1 to approximately 21% and 47%, respectively, on day 21. We conclude that for Atlantic sturgeon semen storage, PC is the extender of choice and semen viability can be maintained for up to 21 d with some expectation of successful fertilization.

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Populations of Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* have decreased drastically owing to overfishing and loss of habitat and as a result this species has been designated as a species of concern by the National Marine Fisheries Service (Atlantic Sturgeon Status Review Team 2007). This led to a significant interest by the U.S. Fish and Wildlife Service (USFWS) and Maryland Department of Natural Resources (MDNR) biologists to establish a gene bank of frozen Atlantic sturgeon spermatozoa (B. Richardson, Maryland Department of Natural Resources, personal communication).

The major hindrance to the ability of biologists to produce Atlantic sturgeon is the inability to have viable gametes of both sexes available simultaneously (Blankenship 2007). During the summer of 2007, the first potentially fertile Atlantic sturgeon female in over 30 years was netted in the Chesapeake Bay (Blankenship 2007). Attempts to artificially spawn this female sturgeon were unsuccessful owing in part to the lack of fertile males. In the absence of any published, efficacious Atlantic sturgeon sperm cryopreservation protocols, and in collaboration with our cooperating agencies, the USFWS, the U.S. Department of Agriculture, and MDNR, we evaluated experimental short-term storage protocols for liquid semen.

Previously, undiluted semen from a single Atlantic sturgeon was stored in 500-mL plastic bags that were replenished daily with oxygen and stored on ice (DiLauro et al. 1994). The sperm sample from this fish, which was supplemented with oxygen daily, retained relatively high motility (80%) and viability (99%) after 5 d of storage. Atlantic sturgeon semen also can be stored on crushed ice and oxygenated daily to extend cell viability (Mohler 2003). Park and Chapman (2005) developed an extender, referred to here as PC, for use in prolonging the storage time for semen of the Gulf sturgeon *A. oxyrinchus desotoi*, a species closely related to the Atlantic sturgeon. Sperm motility in the extended semen was significantly better ($P < 0.05$) than in undiluted semen and was maintained for up to 28 d.

The purpose of this research project was to develop an efficacious, short-term storage protocol for Atlantic sturgeon spermatozoa. Effective short-term storage of semen becomes essential when processing multiple semen samples, or when milt must be transported from collection sites to hatcheries for fertilization of ova or to laboratories for cryopreservation. Because attempts to artificially spawn ripe sturgeon females have been unsuccessful owing to the lack of available spermatozoa, short-term storage provides greater flexibility to fertilize viable eggs from female sturgeon collected at disparate times.

METHODS

Experimental sturgeon and semen collection.—Semen was collected from a total of nine spermiating wild Atlantic sturgeon males captured on their spawning grounds in the Hudson River. A 10-cm length of plastic tubing with 6.4-mm outside diameter (O.D.) was attached to a 60-mL syringe and inserted into the genital opening to extract semen according to accepted methods (Mohler 2003). The area around the urogenital opening was dried thoroughly to prevent the semen from being activated prematurely by contact with water or urine. Semen (25 mL aliquots) was placed into 50-mL sterile tubes and depending on the experiment, either all tubes received oxygen gas before overnight shipment (experiment 1) or half received oxygen gas with the other half receiving air before overnight shipment (experiment 2). The sample tubes were wrapped with a paper towel and placed in a Styrofoam box containing ice packs and then shipped overnight to the University of Maryland Crane Aquaculture Facility (UMCAF).

Semen characterization.—On day 0 (the day that a semen sample arrived at UMCAF), initial screening of semen characteristics of percent motile sperm, osmolality (mOsm/kg), pH, and sperm density (cells/mL of semen) were determined for each sample. Spermatozoa were activated by adding 18 μL of 20 mM Tris-NaCl (80 mOsm/kg, pH 8.0) to 2 μL of semen and then placed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Motility and sperm density were determined from digital recordings made from the activation aliquot with a Magnavox model ZC320MW8 digital recorder (Philips Electronics, Andover, Massachusetts) and a Hitachi Model KP-D20BU high-contrast color digital camera (Hitachi, Tokyo, Japan) attached to a Zeiss Model D-7082 phase-contrast microscope (Carl Zeiss, Berlin, Germany) at 200 × . Osmolality and pH were determined by means of a Westcor Model 5400 vapor pressure osmometer (Westcor, Logan, Utah) and a Hach Model sension2 pH electrode (Hach, Loveland, Colorado), respectively.

Semen samples were collected from a total of 23 males during the 2008 and 2009 spawning seasons. Of the total, 10 were classified as high quality, with initial sperm motility of between 90% and 95% upon arrival to the laboratory. Another 11 samples were of moderate quality, having 43–83% initial motility, and two samples were of poor quality, with 10% and 25% motility. Semen samples of high quality were used from four males in 2008 and five males in 2009 (Table 1) in experiments designed to evaluate short-term storage of Atlantic sturgeon semen and sperm quality over the period of time in storage. Within 1 h

TABLE 1. Initial quality assessment of wild Atlantic sturgeon semen samples on day 0 for experiments 1 (2008) and 2 (2009).

Collection date	Density (10^9 cells/mL)	pH	Osmolality (mOsm/kg)	Motility (%)
Jun 19, 2008	2.2	7.9	75	95
	13.4	7.6	99	90
	3.2	7.6	97	95
	9.3	7.6	90	90
Jun 17, 2009	2.3	7.8	89	92
	2.9	7.7	120	90
	9.7	7.2	104	83
	2.8	7.6	100	90
	12.7	7.6	128	73

of the initial day 0 screening of samples, a subsample of those selected for experiments was placed on ice and transported to the U.S. Department of Agriculture (USDA), Agricultural Research Service, Animal Biosciences and Biotechnology Laboratory at Beltsville, Maryland, for further analysis. Additionally, 1-mL aliquots of each semen sample were assigned to a treatment combination (described in experiments 1 and 2 below) and analyzed at the USDA laboratory every other day for the remainder of the experiment.

Analysis of cell viability.—A flow-cytometric (Epics XL; Beckman-Coulter, Hialeah, Florida) assay that used the fluorescent markers SYBR-14 and propidium iodide (PI) (L-7011; Invitrogen, Carlsbad, California) was employed; this was similar to that published for use in fish by Segovia et al. (2000). The appropriateness of this dual DNA staining combination, for use with sturgeon sperm as well as with other species, was reported a few years later (Flajshans et al. 2004; Guthrie and Welch 2005). Essentially, the marker SYBR-14 permeates the cell membrane and binds with DNA, which is excited by blue laser light to emit green fluorescence. The PI enters only nonviable or membrane-compromised cells and binds to DNA to emit a high-intensity red fluorescence (Garner and Johnson 1995). The percent of cells with green fluorescence was defined as viability and recorded for each sample.

Sperm motion analysis.—Computer-assisted sperm motion analysis was used to determine sperm motion characteristics in 20μm, four-chamber, glass counting slides (SC 20–01 FA; Leja Products, Nieuw-Vennep, The Netherlands) by using a Hamilton Thorne (Hamilton Thorne Biosciences, Beverly, Massachusetts) IVOS version 12 motion analysis system, as previously reported (Vyt et al. 2004; Douglas-Hamilton et al. 2005). The motion analysis program selected and previously described by Guthrie and Welch (2007) identified motile and nonmotile Atlantic sturgeon spermatozoa and was used for all analyses. A 20-mM tris–NaCl solution was used to dilute the samples to a readable concentration (30×10^6 cells/mL) for the IVOS-12 motion analysis system, as well as to initiate activation. Curvilinear velocity (VCL), measured in micrometers per second (μm/s), is the velocity of the sperm head along its actual curvilinear path and was measured simultaneously with motility.

Sperm ATP analysis.—Sperm ATP levels were quantified by means of a luciferin–luciferase assay previously described (Long and Guthrie 2006). Briefly, a 100-μL aliquot of each sample containing 4×10^6 sperm cells was collected after completion of the in vitro treatments and incubated with 1 μL of a 100 × phosphatase–ATPase inhibitor solution (P5728, Sigma-Aldrich, St. Louis, Missouri) for 30 min to inhibit ATP degradation and production before measurement. The extracts were stored at −70°C until they were thawed and boiled to release ATP. The ATP in 10^6 cells was quantified with a luciferin–luciferase assay adapted to measure bioluminescence on a SpectraFluor Plus plate reader (Tecan Group, Maennedorf, Switzerland) and data were recorded as picomoles (pmol) of ATP per 10^6 sperm.

Experimental extenders.—Two experimental extenders previously used to dilute sturgeon semen were evaluated. One was the modified Tsvetkova (MT) medium, which has been used to dilute the sperm of shortnose sturgeon *A. brevirostrum* and pallid sturgeon *Scaphyrinchus albus* (Horvath et al. 2005) before cryopreservation. The other extender was the medium used by Park and Chapman (2005), designated PC, which has been used to dilute the sperm of Gulf of Mexico and shortnose sturgeon. Based on our initial quality assessment of Atlantic sturgeon semen, both extenders were adjusted to a pH of approximately 7.5 and an osmotic pressure of 100 mOsm/kg (D. Guthrie and L. C. Woods III, unpublished data). The compositions of these extenders are shown in Tables 2 and 3.

TABLE 2. Composition of Park and Chapman extender (Park and Chapman 2005). The constituents listed are added to 1 L of sterile, distilled water. Final osmolality ≈ 100 mOsm/kg, pH ∼ 7.5.

Constituent	Amount (g)
Sodium chloride	1.0
Potassium chloride	0.2
Sodium bicarbonate	0.5
Calcium chloride (anhydrous)	0.05
Magnesium sulfate	0.05
Sodium phosphate monobasic	0.15
Sodium phosphate dibasic (anhydrous)	0.15
Sucrose	17.2

Experiment 1.—In 2008, the semen collected from each sturgeon was divided among six sterile 50-mL conical tubes, two each for three experimental dilution treatments: undiluted and diluted 1:3 (one volume semen : three volumes extender) with either the extender MT or PC. Each tube contained 25 mL of undiluted or diluted semen at the beginning of the storage period. One tube of each dilution treatment received oxygen gas as an environmental treatment, while the second tube of a given dilution treatment received nitrogen gas. The oxygen and nitrogen gases used were medical grade products (Airgas, Radnor, Pennsylvania) with 96% and 99% purity, respectively. The storage tubes were kept under refrigeration (4 ± 1°C) in a horizontal position, allowing the majority of cells to remain exposed to the treatment gas. At each sampling period the tubes were opened to allow gases from respiration to evacuate, and a 500-μL aliquot of each sample was removed for the quantitative cell quality analyses described above. The treatment tubes were purged with their respective storage gas, the cells gently

TABLE 3. Composition of modified Tsvetkova solution (Horvath et al. 2005). The constituents listed are added to 1 L of sterile distilled water. Final osmolality ≈ 82 mOsm/kg, pH ∼ 7.5.

Constituent	Amount (g)
Sucrose	8.01
Tris buffer	3.63
Potassium chloride	0.019

resuspended by inverting the tube three times, and tubes were then refrigerated. Samples were analyzed on days 1, 3, 5, and 7. All samples were held over ice during analysis.

Experiment 2.—Based upon the results from the experiment carried out in the 2008 spawning season (experiment 1), storage treatments were modified for the 2009 study. The nitrogen atmosphere was excluded because motility and viability were poor with this treatment. The effect of oxygen was further investigated by comparing the timing and the amount of oxygen added as described below. Air was introduced as an intermediate level of oxygen (21%) between the oxygen and nitrogen atmosphere treatments in experiment 1. The extender PC was superior to MT in experiment 1, so semen was diluted only with PC in experiment 2.

At the time of semen collection, and before shipment to UMCAF, two aliquots of semen were shipped undiluted and two aliquots were diluted 1:3 in PC. The latter were designated as PC extender on the boat (PCB). All samples were transferred into separate, 50-mL conical tubes for shipment. One tube from each pair was purged with oxygen and sealed and the other tube was only sealed (air atmosphere treatment). Upon arrival at UMCAF, two aliquots from the undiluted semen sample treated with air were transferred to two conical tubes and diluted 1:3 in PC. They were designated as PC extender at the laboratory (PCL). One tube of each dilution received one of the two atmospheric gas treatments: oxygen (medical grade, purity 96%) or air. Each of the tubes contained 25 mL of undiluted or diluted semen at the beginning of the storage period. At each sampling period the tubes were opened to allow gases from respiration to evacuate, and a 500-μL aliquot of each sample was removed for the quantitative cell quality analyses described above. The treatment tubes were purged with their respective storage gas, the cells gently resuspended by inverting the tube three times and then tubes were refrigerated. Samples were analyzed on alternate days from day 1 through day 21. Sperm characteristics for each semen treatment were quantitatively evaluated for sperm quality as previously described. All samples were held over ice during analysis.

Statistical analysis.—The experiments were conducted by using a split-split-plot design with dilution treatment applied to whole samples, gas atmospheres to subsamples, and repeated measures across days (modeled as a covariate) comprising sub-subsamples. Statistical analyses were conducted using SAS version 9.2 PROC GLIMMIX (SAS Institute, Cary, North Carolina). Diagnostics of model residuals (Little et al. 1996) indicated the arcsine-square-root transformation of the proportions of viable and motile sperm and the square-root transformation of ATP data were necessary to meet analysis of variance (ANOVA) assumptions. In 2008, the correlation structure among days was modeled as heterogeneous, first-order autoregressive for viability and VCL, as homogeneous, first-order autoregressive for motile, and as heterogeneous, compound symmetric for ATP. In 2009, the correlation structure among days was modeled as heterogeneous, first-order autoregressive for live, motile, and VCL and as heterogeneous, compound symmetric for ATP.

Based upon the results of the PROC GLIMMIX procedures, an appropriate linear regression model of each response variable consisting of the significant main effects or interactions of dilution treatment applied to whole samples and gas atmospheres to subsamples onto day was identified and fit within the single analysis of covariance (ANCOVA) model for that response variable on the transformed scale. Estimates of the linear regression model parameters and comparison of the regression slopes among gases, dilution treatment, or both (when appropriate according to significance of ANCOVA model effects) were obtained with ESTIMATE statements. Pairwise comparisons between gases or among dilution treatments were obtained at each observed day by using PDIFF and LINES options in LSMEANS statements at a significance level of 0.05. Regression curves and 68% confidence limits (CLs) of the predicted response at each observed time, as shown in the figures, and their slopes were obtained from the response variable's ANCOVA model on the transformed scale and then back-transformed to the original scale. On the transformed (i.e., normal) scale, 68% of the data falls within $\pm$ 1 SE from the mean. Back-transformation of these lower and upper 68% CLs produces asymmetric SE bars on the original scale.

RESULTS

Experiment 1

Over the 7-d storage period the significant sources of variation for the linear regression of the arcsine-square-root transformation of the proportion of viable sperm on storage time were the main effects of atmosphere and storage time, the interaction of dilution treatment and storage time, the interaction of atmosphere and storage time, and the three-factor interaction. The back-transformed regression relationships of the six treatment combinations for viability during the 7-d storage period are shown in Figure 1. In the presence of oxygen, sperm viability in undiluted, PC, and MT semen was maintained at a mean of 93% for the 7-d period with no significant regression on day. The day-1 mean values of 90.3% (PC–O_2) and 97.2% (UNC–N_2) differed from each other, but not from the other treatment combination means. In contrast to storage in an oxygen atmosphere, storage in a nitrogen atmosphere was associated with a progressive decline ($P < 0.05$) in sperm viability in PC, MT, and undiluted semen from a mean of 95% on day 1 to mean values of 59, 4.6, and 6.4% on day 7, respectively. During storage under nitrogen, viability based on linear regression coefficients decreased ($P < 0.05$) at a 2.8-fold greater rate in undiluted and MT semen (-17.0%/d for both dilution treatments) compared with PC semen (-6.1%/d).

Significant sources of variation for the linear regression of the arcsine-square-root transformation of the proportion of motile sperm on storage time were the main effects of storage time, the interaction of dilution treatment and storage time, and

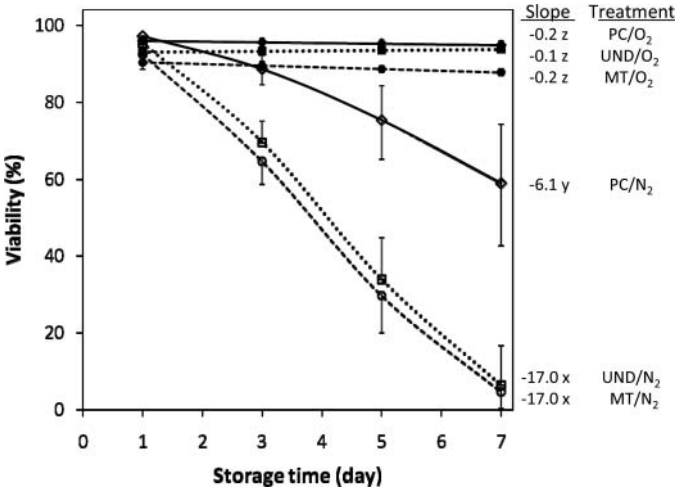

FIGURE 1. Back-transformed regression relationships between the arcsine-square-root transformed proportion of viable Atlantic sturgeon sperm and storage time for the six combinations of dilution treatment and gas atmosphere. Semen collected from wild Atlantic sturgeon during the 2008 spawning season (experiment 1) were stored at 4°C either undiluted (UND) or diluted 1:3 (volume basis) in Park and Chapman extender (PC) or modified Tsvetkova extender (MT) for 7 d in an atmosphere of nitrogen (N_2) or oxygen (O_2). Symbols denote individual data points; error bars denote SEs. The means for the linear regression coefficients (slopes) are also given; means without a common letter are significantly different ($P < 0.05$).

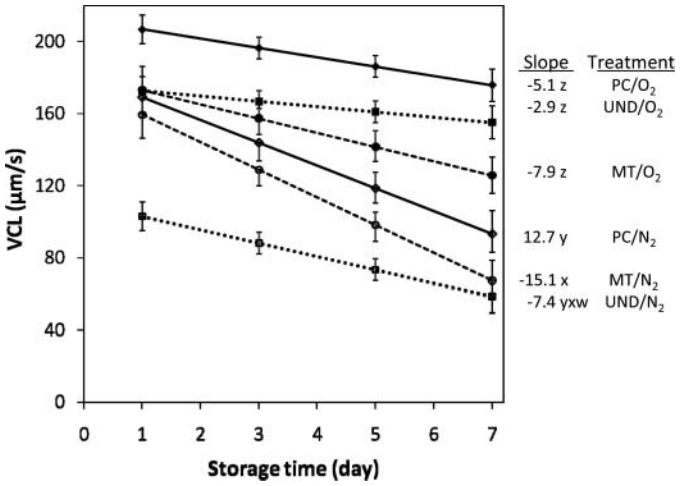

FIGURE 3. Predicted regression relationships between the arcsine-square-root transformed curvilinear velocity (VCL) of Atlantic sturgeon sperm and storage time. See Figure 1 for additional details.

the interaction of gas atmosphere and storage time. The back-transformed regression relationships of the six treatment combinations for motility are shown in Figure 2. The day-1 mean for UND–N_2 of 52.7% was less than ($P < 0.05$) the means for MT–O_2, PC–O_2, and UND–O_2, but did not differ from the means for PC–N_2 and MT–N_2 ($P \geq 0.05$). Motility decreased ($P < 0.05$) during the storage period in the presence of either 100% oxygen or nitrogen atmospheres; however, the rate of decline based on linear regression coefficients was greater ($P < 0.05$) for nitrogen compared with oxygen in PC (−8.6 versus −2.7%/d, respectively), MT (−16.6 versus −3.1%/d, respectively), and undiluted (−10.5 versus −2.0%/d, respectively). There was no significant difference in sperm motility among the dilution treatments incubated under oxygen with motility decreasing ($P < 0.05$) from a mean of 90% on day 1 to 73% on day 7. The differing patterns of decline in motility during storage under nitrogen gas showed the progressively superior protection of motility in undiluted, MT, and PC semen (Figure 2).

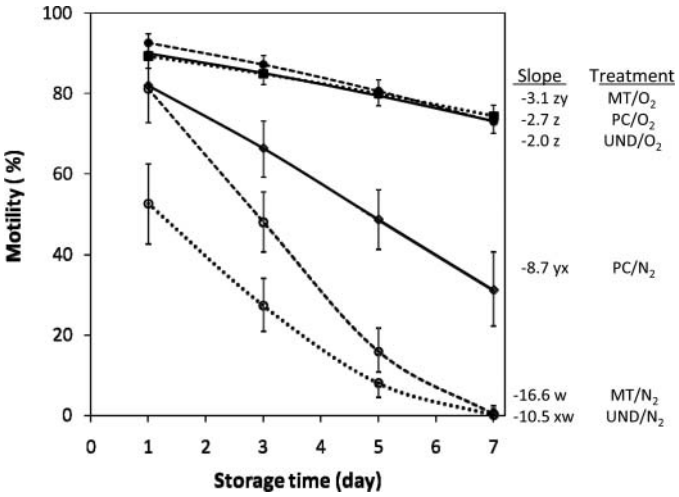

FIGURE 2. Back-transformed regression relationships between the arcsine-square-root transformed proportion of motile Atlantic sturgeon sperm and storage time for the six combinations of dilution treatment and gas atmosphere. See Figure 1 for additional details.

Significant sources of variation for the linear regression of VCL on storage time were the main effects of dilution treatment, gas atmosphere, and storage time and the interactions of dilution treatment and storage time and gas atmosphere and storage time. The linear regression models for the six treatment combinations for VCL are shown in Figure 3. The day 1 mean for PC–O_2, 207 µm/s, was greater ($P < 0.05$) than the mean values for MT–N_2 and UND–N_2, but did not differ ($P \geq 0.05$) from the means for UND–O_2, MT–O_2, and PC–N_2. Sperm VCL decreased during the storage period in the presence of either oxygen or nitrogen atmosphere. The maintenance of sperm VCL was greater ($P < 0.05$) in the oxygen than in the nitrogen atmosphere. The rate of decline in VCL based on linear regression coefficients was two-fold greater ($P < 0.05$) under nitrogen than under oxygen atmosphere for sperm in PC (−12.7 versus −5.1 µm/s per day, respectively) and MT (−15.1 versus −7.9 µm/s per day, respectively), but not different for undiluted semen (−7.4 versus −2.9 µm/s per day, respectively).

Significant sources of variation for the linear regression of the square-root transformation of ATP on storage time were the main effect of storage time and the interaction of atmosphere and storage time. The back-transformed regression relationships of atmosphere on storage time for ATP are shown in Figure 4.

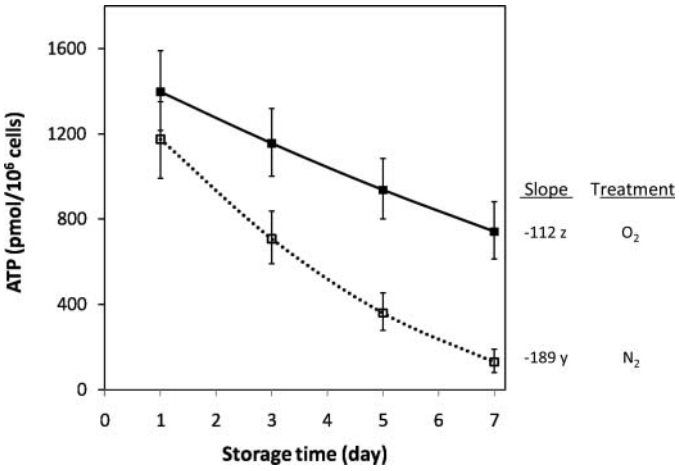

FIGURE 4. Back-transformed regression relationships between the arcsine-square-root transformed concentration of ATP in Atlantic sturgeon sperm and storage time. See Figure 1 for additional details.

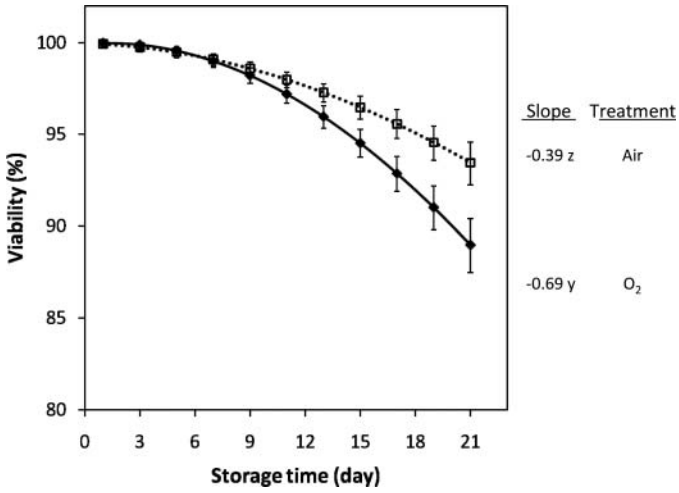

FIGURE 5. Back-transformed regression relationships between the arcsine-square-root transformed viability of Atlantic sturgeon sperm and storage time in an atmosphere of air or oxygen using sperm collected during the 2009 spawning season (experiment 2). See Figure 1 for additional details.

The day-1 mean for O_2 of 1,397 pmol/10^6 cells was greater ($P < 0.05$) than the mean for N_2, 1,175 pmol/10^6 cells. Sperm ATP decreased ($P < 0.05$) during storage under both atmospheres and, based on the linear regression coefficients, decreased ($P < 0.05$) more rapidly under nitrogen than under oxygen, decreasing by a rate of 189 and 112 pmol/10^6 cells per day, a decrease of 46.7% and 88.9% between days 1 and 7 in the presence of oxygen and nitrogen, respectively.

Experiment 2

Over the 21-d storage period significant sources of variation for the linear regression of the arcsine-square-root transformation of the proportion of viable sperm on storage time were the storage time main effect and the interaction of atmosphere and storage time. The back-transformed regression relationships of atmosphere on storage time for viability are shown in Figure 5. Viability was greater than 95% during the first 13 d of storage and then decreased ($P < 0.05$) during the next 8 d to a mean of 94% in the presence of air and 89% in the presence of oxygen. The rate of decline in viability based on linear regression coefficients was greater ($P < 0.05$) for storage under an oxygen than under an air atmosphere (−0.69 versus −0.39%/d, respectively).

Sources of variation for the linear regression of the arcsine-square-root transformation of the proportion of motile sperm on storage time were the storage time main effect and the interaction of dilution treatment and storage time. The back-transformed regression relationships of dilution treatment on storage time for motility are shown in Figure 6. Atmosphere had no effect on sperm motility ($P > 0.05$). Motility was 86% on day 1 and decreased ($P < 0.05$) for the three dilution treatments during the course of the experiment. Based on the linear regression coefficients the pattern of change was similar for the three dilution treatments, except motility decreased ($P < 0.05$) at a greater rate in undiluted semen than in PCB and PCL semen (−3.49, −2.20, and −1.86%/d, respectively).

The only significant source of variation for VCL was the linear regression on storage time. The mean VCL was 119 μm/s on day 1 and decreased at a rate of 1.68 μm/s per day (SE = 0.16) over the remainder of the experiment (data not shown). Over the 21-d storage period the only significant source of variation for the square-root transformation of ATP content was the linear regression on storage time ($P < 0.05$). Sperm ATP increased from 855 to 1,125 pmol/10^6 cells between days 1 and 21 (data not shown).

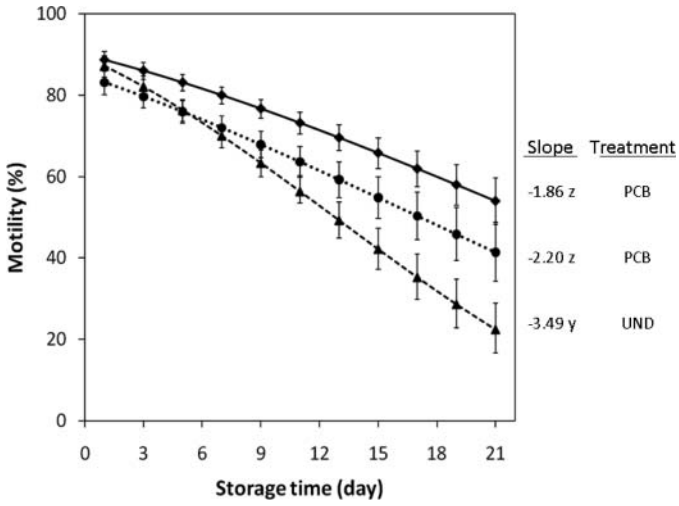

FIGURE 6. Back-transformed regression relationships between the proportion of motile Atlantic sturgeon sperm and storage time. Semen collected from wild Atlantic sturgeon during the 2009 spawning season (experiment 2) were stored at 4°C either undiluted (UND) or diluted 1:3 (volume basis) in Park and Chapman extender at the time of collection (PCB) or after arrival in the laboratory (PCL) and stored for 21 d in an atmosphere of air or oxygen. See Figure 1 for additional details.

DISCUSSION

Semen to be stored for short periods of time, or indefinitely when cryopreserved, is most often extended with saline solutions that mimic the osmotic pressure found in the seminal plasma (Fuller et al. 2004). Billard et al. (2004) reviewed the short-term storage of sturgeon semen and reported that the simplest approach was to store the semen chilled and undiluted. Ciereszko et al. (1996) stated that sturgeon sperm stored chilled on ice for 3 d have "similar properties to fresh semen" and that acrosin-like activity of lake sturgeon *A. fulvescens* could be maintained for up to 13 d. DiLauro et al. (1994) collected semen from a single male Atlantic sturgeon captured from the Hudson River and reported that the semen, kept chilled and replenished with oxygen daily, "may be kept in a motile and viable state for at least 5 d." Mohler and Fletcher (1999) later reported that sperm, collected from an Atlantic sturgeon male captive for 4 years and held in oxygenated bags kept chilled on ice, had motility comparable with fresh sperm collected from wild males for 2 d. Park and Chapman (2005) reported that semen collected from both the Gulf of Mexico sturgeon and the shortnose sturgeon and stored undiluted deteriorated within hours if stored at room temperature, and even when stored under refrigeration, the semen lost viability within 1 week. Billard et al. (2004) noted that extenders, when used to dilute sturgeon semen, varied but were often either simple sugar or simple saline-based solutions buffered with tris-HCl ($\leq$150 mM). Glogowski et al. (2002) used a tris-buffered sucrose solution, in combination with either NaCl (25 mM) or KCl (0.25 mM) to extend Siberian sturgeon *A. baerii* semen successfully for 2 h before cryopreservation. Linhart et al. (1995) reported that they used a simple sugar solution (100–150 mM glucose, 20 mM tris) to effectively extend the semen from shovelnose sturgeon *S. platorynchus*. Brown and Mims (1995) developed a simple saline extender for paddlefish *Polyodon spathula* containing only sodium chloride and antibiotics that provided a fertilization rate of 97% after 14 d of cold storage. Based on a careful analysis of the seminal plasma Park and Chapman (2005) were able to develop a more complex extender for the Gulf of Mexico sturgeon that maintained motility at approximately 45% after 21 d of refrigerated storage compared with no motility in undiluted semen at 21 d.

Oxygen was identified as an important factor in the short-term storage of Atlantic sturgeon semen by Conte et al. (1988) and DiLauro et al. (1994), who replaced the oxygen every 12 and 24 h, respectively, in their studies. Storage of sperm in an oxygen atmosphere was superior to storage under nitrogen for all sperm characteristics measured during experiment 1, and the presence of oxygen equalized the responses for viability in undiluted semen and semen diluted in PC or MT in the 2008 season. During the 2009 season, sperm stored in PC at the time of collection or in the laboratory under 100% oxygen or air maintained viability at more than 90% through 13 d and then diverged to 94% and 88%, respectively, on day 21. Motility of sperm diluted in PC and stored under air or oxygen at the time of collection or in the laboratory did not differ significantly during the 21-d storage period. We hypothesized that an oxygen-rich environment would be necessary for the cells to survive and remain of high quality, because oxygen is essential for aerobic respiration in sperm cells. Compared with 100% oxygen, it appears that the storage in air supplied sufficient oxygen (21%) to maintain sperm viability and motility.

Semen dilution may mitigate the harmful effects of longer storage periods by reducing the chances of any potential contamination by water or urine at collection to protect cells from premature motility activation or bacterial contamination (Park and Chapman 2005). Because the Gulf sturgeon and Atlantic sturgeon are closely related subspecies, the positive, significant effects of using the PC extender may be due to the fact that they share similar seminal plasma characteristics. The PC extender composition was based on measured concentrations of sodium chloride, glucose, and potassium in the seminal plasma of the Gulf sturgeon and the extender was based on those measurements (Park and Chapman 2005).

In addition, the presence of glucose and phosphate may be an important advantage of PC over MT. Glucose and inorganic phosphate would be important to support the energy status of sturgeon sperm cells because it is possible that sturgeon sperm, like those of mammalian species, possess glucose pores (Sancho et al. 2007; Kim and Moley 2008) and transporters for inorganic phosphate (Zarca et al. 1988). If so, glucose could be used for glycolytic ATP production and as a substrate for utilization in the Krebs citric acid cycle to generate NADH and $FADH_2$ for powering oxidative phosphorylation to produce additional ATP. The sperm cell's need for oxygen has been reported by Billard et al. (2004) to affect ATP stores, which can become depleted when cells are held in anoxic conditions, leading to a decline in cell motility. While there was a measureable difference in the progression of ATP content in sperm stored under oxygen between the two experiments, we found a decrease in ATP during 2008 and an increase during 2009 using the same ATP analysis methodology. We have no explanation for this difference.

The extender MT has been primarily used as an extender for sturgeon semen cryopreservation (Zarca et al. 1988). We have compared MT and PC containing dimethyl sulfoxide as cryoprotective extenders for Atlantic sturgeon cryopreservation and found that after thawing neither extender provided adequate viability or motility (D. Guthrie and L. C. Woods III, unpublished). Therefore, our results using short-term liquid storage with the PC extender support the use of this extender or diluent under refrigeration as an alternative to cryopreservation.

We conclude that under these conditions of Atlantic sturgeon semen storage, PC is the extender of choice and semen can be stored under oxygen or air for up to 21 d with some expectation of fertility. The results of these studies should allow for better management of increasingly scarce Atlantic sturgeon gametes. However, fertility trials will be required to confirm this point. Semen stored for various periods of time should be tested for its ability to fertilize Atlantic sturgeon eggs, or in the absence of available Atlantic sturgeon eggs, develop a sperm penetration

or fertilization assay with eggs of other sturgeon species to evaluate the ability of the stored Atlantic sturgeon sperm to reach the oolemma, exhibit an acrosome reaction, and penetrate the oolemma.

With effective techniques, biologists will not only be better supplied with the gametes necessary to generate new recruits for the diminishing Atlantic sturgeon populations whenever limited opportunities arise but will also be able to conserve important genetic variation obtained from the remaining Atlantic sturgeon populations. These results may also prove useful as initial cryopreservation protocols for the Atlantic sturgeon are developed.

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