

ABSTRACT

Title of Thesis: QUALITY ASSESSMENT OF ATLANTIC STURGEON
 (*ACIPENSER OXYRINCHUS*) SPERMATOZOA UNDER
 CONDITIONS OF SHORT-TERM STORAGE

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Short-term storage trials were conducted in 2008 and 2009 on Atlantic sturgeon semen obtained from captive males, held at the U.S. Fish and Wildlife Service, Northeast Fish Technology Center, that were hormonally induced to spermiate; and wild males collected during the spawning season from the Hudson River. Samples were stored under refrigeration ($4 \pm 1^{\circ}\text{C}$) in treatments consisting of different gaseous environments (oxygen, nitrogen or air) and experimental diluents (Modified Tsvetkova extender, Park & Chapman extender and neat, i.e. undiluted). Analyses of gamete quality were performed on day 0 (pre-treatment), and then every other day for 7 days in 2008 and for 21 days in 2009. Sperm quality parameters evaluated included: viability, motion analysis, curvilinear velocity and cellular ATP levels. Higher gamete quality was maintained when spermatozoa were diluted in the PC extender and stored in the presence of oxygen.

QUALITY ASSESSMENT OF ATLANTIC STURGEON (*ACIPENSER OXYRINCHUS*)
SPERMATOZOA UNDER CONDITIONS OF SHORT-TERM STORAGE

By

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INTRODUCTION

The Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) is one of the world's most primitive fish species ranging from New Brunswick, Canada to the eastern coast of Florida. They are an anadromous fish, migrating upstream freshwater tributaries during spring and early summer to spawn. Atlantic sturgeon can live up to 50 years, but are a late-maturing fish that take anywhere from seven to twenty-eight years to sexually mature (Mohler, 2003). Typically, Atlantic sturgeon can reach lengths of 3 to 4 meters and may weigh in excess of 360 kilograms. They possess bony plates, called scutes, rather than scales. They have a long snout and their coloration ranges from black to grey to olive green. They feed on benthic organisms like amphipods, worms, and shrimp, and are known for their unusual jumping behavior (Mohler, 2003). This unique jumping behavior is thought to be a form of communication to maintain group cohesion (Sulak et al., 2002).

Atlantic sturgeon, now a Species of Concern (NMFS & NOAA, 2004), were once very abundant throughout their range. Because their meat and caviar have a high commercial value, Atlantic sturgeon populations have decreased drastically due to overfishing. Subsequently, populations have been unable to recover, which along with a reduction in the number of spawning adults in the wild and a late age of sexual maturation, has resulted in recommendation for listing as endangered or threatened (Atlantic Sturgeon Review Team, 2007). In 1998, a 20 to 40 year moratorium was enforced on all Atlantic sturgeon fisheries. This moratorium will remain in place until spawning stocks on the Atlantic coast have been restored or at minimum of 20 year classes of adult females have been protected (Mohler, 2003).

There has been a significant interest by the U.S. Fish and Wildlife Service (USFWS) and natural resource agency biologists in establishing a gene bank of frozen Atlantic sturgeon spermatozoa (Brian Richardson of the Maryland Department of Natural Resources, personal communication). The major hindrance to the ability of biologists to produce Atlantic sturgeon is the inability to have ripe fish of both sexes available simultaneously (DiLauro et al., 1994). During the summer of 2007, the first ripe Atlantic sturgeon female was netted in Chesapeake Bay in over thirty years (Blankenship, 2007). Attempts to artificially spawn this female were unsuccessful due in part to the lack of available and appropriate spermatozoa (Richardson et al. 2007). To date, no Atlantic sturgeon eggs have been successfully fertilized *in vitro* using cryopreserved milt. Consequently, our cooperating agencies (USFWS, United States Department of Agriculture, and Maryland Department of Natural Resources) are initially more interested in a viable short-term storage protocol, rather than cryopreserved semen.

DiLauro et al. (1994) stored neat semen from a single Atlantic sturgeon male in 500mL plastic bags replenished daily with oxygen and stored on ice. Sperm from this fish sample supplemented with oxygen daily retained relatively high motility (80%) and viability (99%) after 5 days of storage. Mohler (2003) also recommended that Atlantic sturgeon semen be stored in a container on crushed ice and oxygenated daily to extend its viability.

Park and Chapman (2005) developed an extender (PC) for use in prolonging the time that semen can be stored of a closely related species to the Atlantic sturgeon, the Gulf sturgeon (*Acipenser oxyrinchus desotoi*). The ingredients of the extender were determined based on the osmolality, pH, sodium chloride (NaCl), potassium (K^+) and

glucose (C₆H₁₂O₆) concentrations in the seminal plasma of male Gulf sturgeon. Semen was diluted with the extender 1:3 (one part semen in three parts extender), placed on an orbital shaker kept in a refrigerator and evaluated after 1, 3, 7, 14, 21 and 28 days of storage. Sperm motility in the extended semen was significantly better ($p < 0.05$) than neat semen and was maintained for up to 28 days (>55%). However, no significant differences were seen in fertilization ability between neat and extended semen (65% and 30%, respectively).

Horvath et al. (2005) successfully cryopreserved shortnose sturgeon (*Acipenser brevirostrum*) semen diluted 1:1 using a Modified Tsvetkova's extender (MT) with 5, 10 or 15% DMSO or methanol. They determined an average cooling rate of 66°C/min and 70°C/min for DMSO and methanol, respectively to be optimum for their cryopreservation protocol.

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 laboratories for cryopreservation. Biologists must have mature gametes from both genders simultaneously to artificially spawn Atlantic sturgeon. Because attempts to artificially spawn ripe females have been unsuccessful due to the lack of available spermatozoa, short-term storage provides greater flexibility to fertilize viable eggs from female sturgeon collected at disparate times.

The goal of the study was to improve conditions of the seminal environment to support and maintain a high percentage of quality spermatozoa. It was hypothesized that

an oxygen-rich environment would be necessary for spermatozoa to survive and remain of high quality, because oxygen is essential for aerobic respiration in sperm cells. During the first year various quality attributes of Atlantic sturgeon semen were compared after being stored with and without oxygen and either undiluted or in two different extenders over a period of seven days. Extenders are solutions made up of salts and other organic compounds and used because they may extend the life of sperm cells while semen is stored. Semen samples are diluted in extenders that are chosen based on their ability to hold the cells quiescent and prevent premature activation which can deplete the cell's energy supply.

In year two, certain treatments used in year one were used again while expanding the storage period. Treatments proven to be efficacious in year one were used in year two to help determine the best possible short-term storage protocol. Samples were exposed to either oxygen gas or air and either stored undiluted or diluted with the PC extender. For the portion of a given fish semen sample that was to be diluted, half of the volume would be diluted immediately at the time of semen collection, while the remaining volume would not be diluted until approximately one day post-collection when they arrived at the lab. In addition, samples were carried out to 21 days, as opposed to 7 days as in year one, and gamete quality was assessed over this increased storage period.

The results of these studies should allow for better management of increasingly scarce Atlantic sturgeon and their collected gametes. It is anticipated that with efficacious storage protocols, biologists will not only be better equipped to generate new recruits for the sturgeon populations as spawning opportunities arise, but will also be able to

conserve important genetic variation of remaining Atlantic sturgeon through efforts to cryopreserve germplasm when not needed for fertilization.

MATERIALS AND METHODS

YEAR-ONE

Wild males were captured and semen collected from mature males (n=11) on the spawning grounds in the Hudson River. Captive males (n=7) held at the USFWS Fish Technology Center, Lamar, Pennsylvania were administered a 1mg/kg injection of common carp pituitary (CCP), to induce spermiation, intramuscularly in soft tissue between the dorsal scutes (Mohler, 2003).

Testes were catheterized to extract semen according to accepted methods (Mohler, 2003). Briefly, the area around the uro-genital opening was dried thoroughly to prevent the semen from being activated prematurely by contact with water or urine. A 60cc syringe attached to 6.4mm O.D. of plastic tubing was used to extract milt (Figure 1). Collected semen (25mL aliquots) was placed into 50mL sterile tubes to which oxygen was added. 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).



Figure 1. Catheterization of testis to collect from a hormone-induced male Atlantic sturgeon (Mohler, 2003).

On day 0 (day that semen sample arrives at UMCAF), each individual's semen sample ($\geq 100\text{mL}$) was checked for sperm motility (%), osmolality (mOsm/kg), pH, and sperm density (number of cells per mL of semen). Initial motility was measured by placing $2\mu\text{L}$ of sturgeon semen into a Makler counting chamber positioned under a light microscope and then adding $18\mu\text{L}$ of 20mM Tris-NaCl (pH 8.0; 80mOsm) to activate the spermatozoa. The activated sample was digitally recorded for later review for the determination of motility as well as the sperm density. If semen was of high initial quality (day 0 cell viability $\geq 90\%$), the sample was used in experiments designed to enhance the short-term storage of the spermatozoa for conservation of the germplasm. Quality semen also exhibited sperm cells moving vigorously in a straightforward motion for at least 30 seconds, followed by a gradual decrease in activity. Only three of the captive samples and four of the wild samples met the *a priori* quality standards and were used in this study.

Osmolality was measured using a Wescor 5520 vapor pressure osmometer and pH was measured using a Hach SensIon2. Prior to applying treatments to the samples, a 1mL aliquot of the pre-treated semen was taken to Beltsville Agricultural Research Center's (BARC) Animal Biosciences and Biotechnology Laboratory for day 0 baseline quantitative gamete quality analyses.

Sperm quality was quantitatively evaluated for the percentage of viable and motile cells, curvilinear velocity and cellular energy. In order for samples to be quantitatively analyzed, their cell density had to be adjusted to approximately 30×10^6 cells/mL for both the computer motion and flow cytometric analyses.

An assay using the fluorescent markers Sybr-14 and propidium iodide (PI) was used to determine the viability of spermatozoa (Figure 2). To each treatment tube, 4µl of Sybr-14 and 0.4µL of PI was added, which were then immediately analyzed using flow cytometry. Sybr-14 permeates through the cell membrane, binding with DNA, which effectively stains viable cells green. Cells stained red by the membrane impermeable stain PI, indicates damage to the plasma membrane and thus, are considered non-viable. The flow cytometer used was an Epics XL, Beckman-Coulter Co., Hialeah, FL, USA.

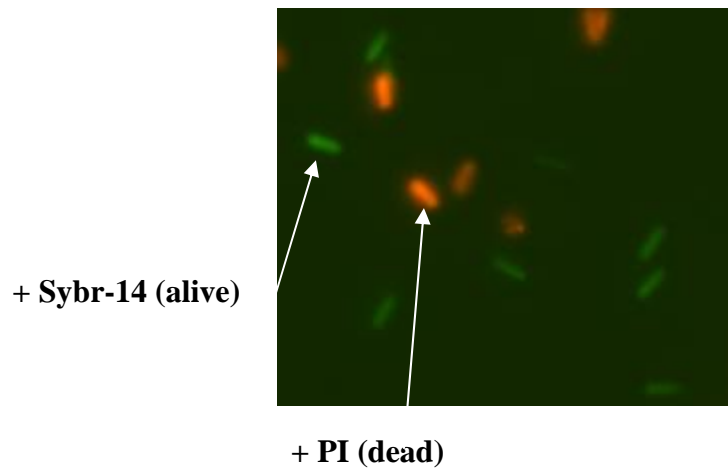


Figure 2. Viable sperm cells are stained green with Sybr14 while non-viable cells are stained red with propidium iodide.

An IVOS-12, Hamilton Thorne computer assisted sperm motion analysis system (CASA) was used to determine motility characteristics of the spermatozoa. Cells were diluted with a 20mM Tris-NaCl (pH 8.0; 20mOsm; Table 1) solution to bring down the density to a readable concentration for the CASA as well as to initiate motility. Quality semen exhibited sperm cells moving vigorously in a straightforward motion for at least 30 seconds, followed by a gradual decrease in activity.

Curvilinear velocity (VCL), measured in micrometers per second ($\mu\text{m/s}$), is the velocity of the sperm head along its actual curvilinear path. VCL was measured simultaneously with motility using the CASA. This assay is important because it characterizes a functional aspect of the sperm. Sperm cells move in an abnormal, erratic circular pattern if damage has been done to the flagellum. Thus, VCL gives a measure of

the normal functionality of the sperm cells. In addition, cells will lose speed with the progression of time.

Mitochondria in a cell produce chemical energy, as adenosine triphosphate (ATP), which is necessary for sperm motility. Thus, the assay described below is useful for assessing sperm function. ATP levels in the sperm cells were quantified using the firefly luciferin-luciferase assay on a SpectraFluor Plus plate reader (Tecan Group Ltd., Maennedorf, Switzerland). The specific assay used was that published by Long & Guthrie (2006), who validated an ATP assay that is capable of processing multiple samples. Luciferin is a competitive inhibitor of firefly luciferase. Oxidation of luciferin, involving ATP, is catalyzed by the enzyme luciferase which results in the production of light. The intensity of the emitted light is proportional to the ATP concentration of the cell, thus the bioluminescence measured by the SpectraFluor Plus plate reader, gives a measure of the energetics of individual semen samples.

Two experimental extenders previously used to dilute sturgeon semen were evaluated. The first was the MT extender, which has been used to dilute sperm prior to cryopreservation of shortnose (*Acipenser brevirostrum*) and pallid (*Scaphyrinchus albus*) sturgeon (Horvath et al., 2005) and the second was PC, used to extend Gulf of Mexico (*Acipenser oxyrinchus desotoi*) and shortnose (*Acipenser brevirostrum*) sturgeon (Park & Chapman, 2005). Because the captive fish semen samples were received one week prior to those of the wild fish in year one, and also because we were unaware of the Park & Chapman publication during captive semen sample collection, only the MT extender was used in the study with the captive fish. Both the MT and PC extenders were used as

diluent treatments with the wild fish semen. Both extenders were adjusted to a pH of 8.0 and an osmotic pressure of 80mOsm (Table 1).

Each individual semen sample was divided into 25mL aliquots, for two gaseous storage treatments: oxygen and nitrogen, or the absence of oxygen (Figure 3). Each treatment aliquot was placed into a sterile, 50mL conical tube under the given treatment gas. Subsamples in each gas environment were additionally subdivided into three experimental dilutions: the two experimental extenders: MT and PC, as well as undiluted semen or “neat.” The MT and PC extended samples were diluted 1:3 (one part semen: three parts extender).

After each 24 hour period of refrigerated storage, the tubes were opened to allow gases from respiration to evacuate. A 500 μ L aliquot of each treatment was then collected for the quantitative cell quality analyses described below. The treatment tubes were purged with their respective storage gas, the cells gently resuspended by inversion of the tube three times and then placed back under refrigeration. The storage tubes were kept under refrigeration ($4 \pm 1^{\circ}\text{C}$) in a horizontal position, allowing the majority of cells to remain exposed to the treatment gas. Samples were then analyzed on days 1, 3, 5 and 7. All samples were held over ice during analysis.

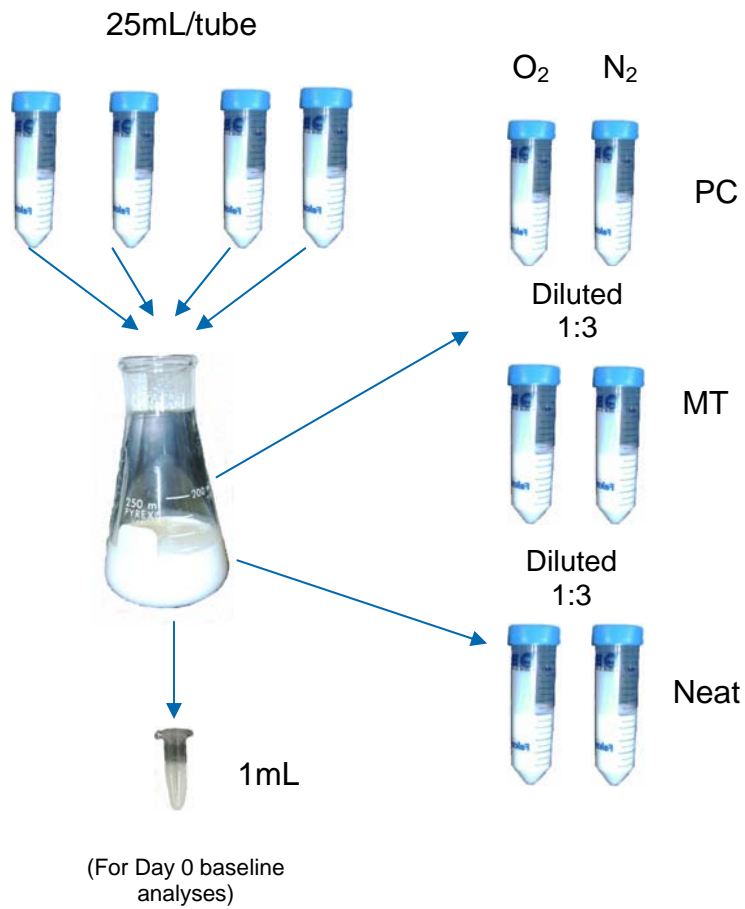


Figure 3. Year-One experiment. The top four tubes (upper left) are indicative of one fish's total semen sample (approximately 100mL). Each sterile tube was filled half way (25mL) with semen to allow exposure of the cells to the gaseous environment. Sample treatments included presence or absence of oxygen and either undiluted (Neat) or diluted semen using the experimental extenders: Park and Chapman (PC) or Modified Tsvetkova (MT). NOTE: The PC extender was not a treatment in the domestic fish study.

An additional preliminary study was performed to examine the potential benefit of using antibiotics with Atlantic sturgeon semen when stored for up to 21 days. A solution of penicillin-streptomycin (5000 units of penicillin, 5mg of streptomycin per mL of 0.9% NaCl sterile water) that has been commonly used for this purpose in other fish species (Brown and Brown, 2000) was added to half (n = 3) of the sample replicates, at the beginning of the experiment and again at the beginning of each of the subsequent two weeks of the study. The remaining half (n = 3) of the semen aliquots, were used as the control and did not receive the antibiotics. It was hypothesized that treating samples with antibiotics would control bacterial growth and have a positive effect on gamete quality over the 21 day study. Samples were exposed to oxygen and subdivided into the same three experimental dilutions as in the year one storage study: MT or PC and neat or undiluted. The MT and PC extended samples were diluted 1:3, just as in our short-term storage experiment. All samples were processed just as previously described in the main storage study. All semen treatments were similarly analyzed quantitatively for gamete quality as previously described, but for every other day for 21 days.

YEAR-TWO

Just as in year one, samples were obtained from both wild and captive males. Wild males were captured and semen collected from mature males (n=12) on the spawning grounds of the Hudson River. Captive males (n=5) held at the USFWS Fish Technology Center, Lamar, Pennsylvania were administered a 0.03 mg/Kg injection of LHRH analog (D-ala⁶-des-Gly⁵LHRH ethylamide) to induce spermiation, intramuscularly in soft tissue between the dorsal scutes (Mohler, 2003). Only four of the captive samples and five of the wild samples met our *a priori* quality standards and were used in this study. The semen collection tubes were wrapped in paper towels and placed in a Styrofoam box containing ice packs and then shipped overnight to the University of Maryland Crane Aquaculture Facility (UMCAF).

Year-two's treatments were chosen amongst the most favorable treatments from year one: semen diluted with the PC extender and stored under oxygen; as well as additional treatments (Figure 4). Semen samples were stored for up to 21 days. All samples were processed upon arrival to the lab as previously described in the first year of study.

Each semen sample was divided into three experimental dilutions: neat, dilution with PC at the time of collection (PCB), and dilution with PC upon arrival to the lab (PCL). All samples were diluted 1:3 (one part semen: three parts PC extender) as described by Park & Chapman (2005). Samples were then subdivided for two gaseous storage treatments: oxygen and air. Air replaced the nitrogen gas treatment from year one's studies, as air represents an intermediate level of oxygen between the oxygen and nitrogen treatments examined in year one. Samples were analyzed on Days 1, 3, 5, 7, 9,

11, 13, 15, 17, 19 and 21. All semen treatments were quantitatively evaluated for gamete quality as previously described. All samples were held over ice during analysis.

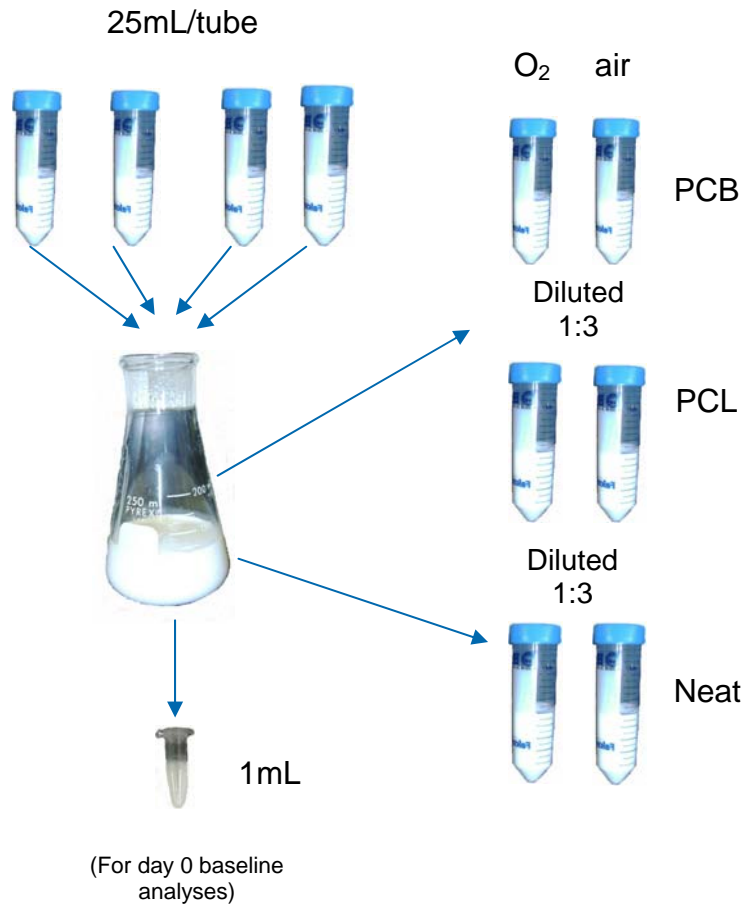


Figure 4. Year-two experiment. The top four tubes (upper left) are indicative of one fish's total semen sample (approximately 100mL). Each sterile tube was filled half way (25mL) with semen to allow exposure of the cells to the gaseous environment. Sample treatments include: presence of oxygen or air; no dilution (Neat); or PC-diluted, either at time of collection on the boat (PCB) or upon arrival to the lab (PCL).

STATISTICAL ANALYSIS

Short-term Storage Studies Conducted During Year One and Year Two

Comparisons of an individual's sperm quality over time were made to the baseline values along with comparisons between treatments. Individual fish served as replicates and experimental units were semen samples treated with a diluent and gas. All data were expressed as means \pm standard error of the mean (SEM). Statistical analyses were done using SAS version 9.2 (SAS Institute Inc. 2003, Cary, NC, USA). Specifically, repeated measures ANOVA was used to account for correlation between repeated measures on the same semen sample. Differences between treatment means were identified using Tukey's multiple comparison test. Factors included gas and diluent. In year one, the levels of diluent included neat, MT and P and the levels of gas included oxygen and nitrogen. In year two, levels of diluent included neat, dilution with PC immediately upon collection (PCB) and dilution with PC one day post-collection upon arrival to our facility (PCL) and the levels of gas included oxygen and air. Residuals were tested for normal distribution, and when not observed, data were logarithmically transformed. P-values <0.05 were considered to be significant.

Antibiotic Study Conducted During Year One

Results of the 21 day antibiotic trial were assessed by one-way ANOVA. P-values <0.05 were considered to be significant. All data, was checked for normality using SAS version 9.2. Viability and motility were the only two variables measured. Factors included diluent and antibiotics. The levels of diluent included neat, MT or PC and the levels of antibiotics included either no addition or the addition of antibiotics.

RESULTS: YEAR ONE

DAY 0 QUALITY ASSESSMENT

The initial quality of semen samples upon arrival to the lab, or day 0, for both captive and wild Atlantic sturgeon obtained during the 2008 spawning season varied (Table 2, Appendix), especially between the captive and wild populations. Although not statistically tested, there was an apparent variation in the pH and initial motility of captive sturgeon semen samples when compared to the wild samples. Sperm density varied more within the wild semen samples. Osmolalities of captive semen samples varied from 66-113mOsm (mean = 82.5mOsm), motilities varied from 0-95% (mean = 61.3%) and the pH varied from 7.4-8.4 (mean = 7.88). Osmolalities of wild semen samples ranged from 75-150mOsm (mean = 110mOsm), motilities varied from 50-95% (mean = 77.3%) and the pH ranged from 7.2-7.9 (mean = 7.49).

SHORT-TERM STORAGE STUDY

All residuals had normal distribution, with the exception of the ATP, which was logarithmically transformed to account for lack of normality of residuals. There was a significant gas by diluent interaction across all variables measured in both captive and wild fish. Because of these significant interactions, significant main effects were ignored, and simple effects were discussed.

WILD STURGEON

At the end of seven days, viability was highest when samples were treated with oxygen than samples treated with nitrogen. In the absence of oxygen, the percentage of viable cells was highest when semen was diluted with the PC extender, compared to undiluted semen or samples diluted with the MT extender (Figure 5).

At the end of the seven day storage period, motility was highest when samples were stored in the presence of oxygen. In the absence of oxygen, the percentage of motility was significantly higher when semen was diluted with the PC extender, compared to the undiluted semen or samples diluted with the MT extender (Figure 6).

At the end of the seven days, VCL of undiluted samples and samples diluted with PC was significantly higher than all other treatments. VCL of samples diluted with MT in the presence of oxygen was significantly higher than all nitrogen treatments, but not better than undiluted or PC-diluted samples stored in the presence oxygen. However, VCL of samples diluted with PC in the absence of oxygen was significantly higher than the undiluted and MT-diluted samples exposed to nitrogen (Figure 7).

Cellular energy remained highest when undiluted or diluted (both MT and PC) samples were stored in the presence of oxygen. However, cellular energy levels in samples diluted with PC and stored in the absence of oxygen were significantly higher than the other two nitrogen treatments (Figure 8).

Results from the wild fish showed that over a seven day storage period in the presence of oxygen, sperm quality was highest when semen was either undiluted or diluted with the PC extender. Furthermore, in the absence of oxygen sperm quality was

significantly higher when semen was diluted with the PC extender. Based on these conclusions, we included the neat and PC extender as well as the oxygen treatment in year two's study, along with some additional treatments.

Effect of O₂ vs. N₂ and Diluent on Viability of Wild Atlantic Sturgeon Spermatozoa

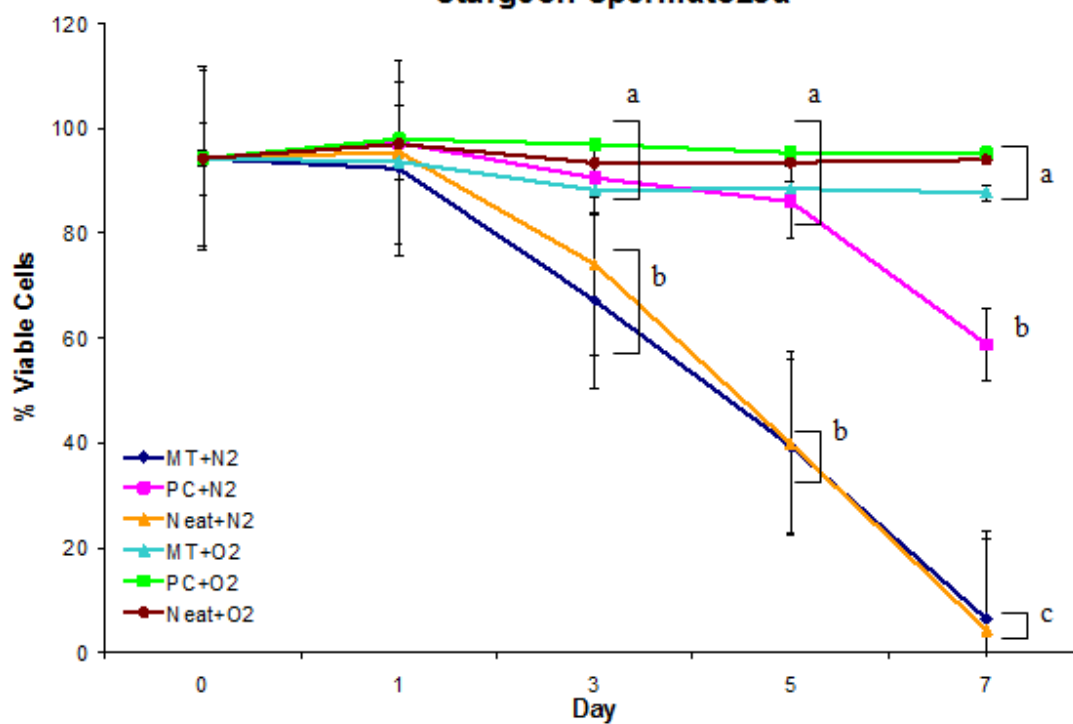


Figure 5. Viability of wild Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on Motility of Wild Atlantic Sturgeon Spermatozoa

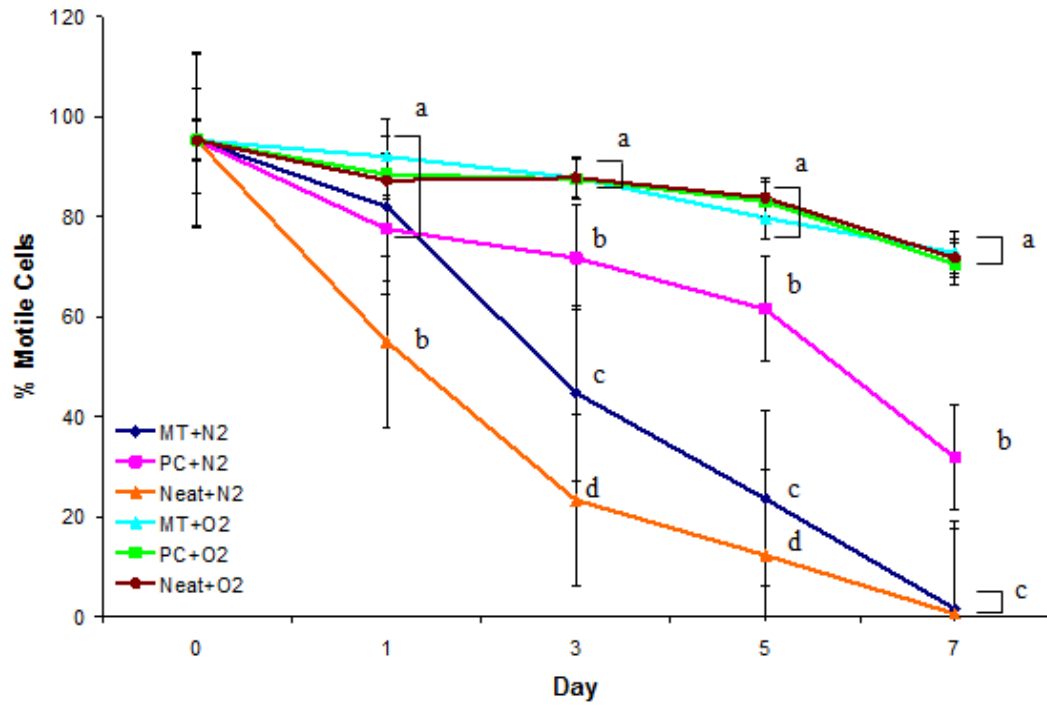


Figure 6. Motility of wild Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on Curvilinear Velocity of Wild Atlantic Sturgeon Spermatozoa

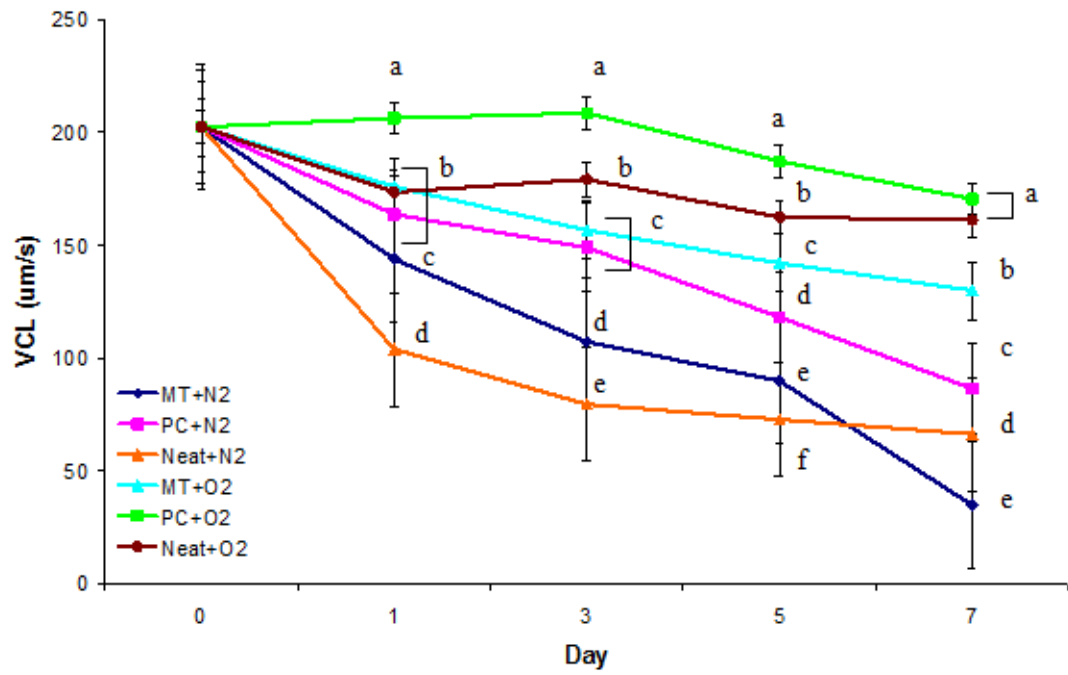


Figure 7. Curvilinear velocity of wild Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on ATP content of Wild Atlantic Sturgeon Spermatozoa

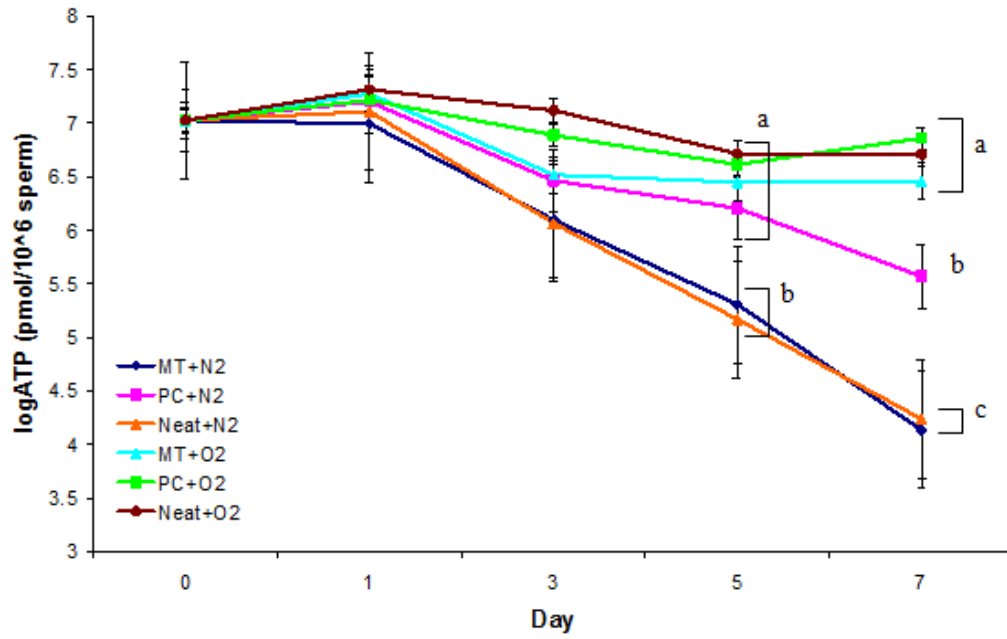


Figure 8. ATP content of wild Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

CAPTIVE STURGEON

At the end of the seven day storage period, viability was highest when neat samples were treated with oxygen. Also, after 7 days the percentage of viable cells was highest in both gas treatments when diluted with the MT extender, compared to the undiluted samples treated with nitrogen (Figure 9).

At the end of seven days, motility was highest when semen was undiluted and stored in the presence of oxygen. Motility was significantly lower in both MT-diluted samples and undiluted samples stored in the absence of oxygen (Figure 10).

VCL remained highest when neat samples were treated with oxygen. VCL of MT-diluted samples in the presence and absence of oxygen was significantly better than quality of undiluted samples exposed to nitrogen (Figure 11).

At the end of the seven day storage period, cellular energy remained highest when undiluted samples were stored in the presence of oxygen. Also, ATP content was highest when samples were diluted with MT in the presence and absence of oxygen than undiluted samples in the absence of oxygen (Figure 12).

Results of the captive data indicate leaving the samples neat and adding oxygen is the best method for storing Atlantic sturgeon semen for up to seven days.

Effect of O₂ vs. N₂ and Diluent on Viability of Captive Atlantic Sturgeon Spermatozoa

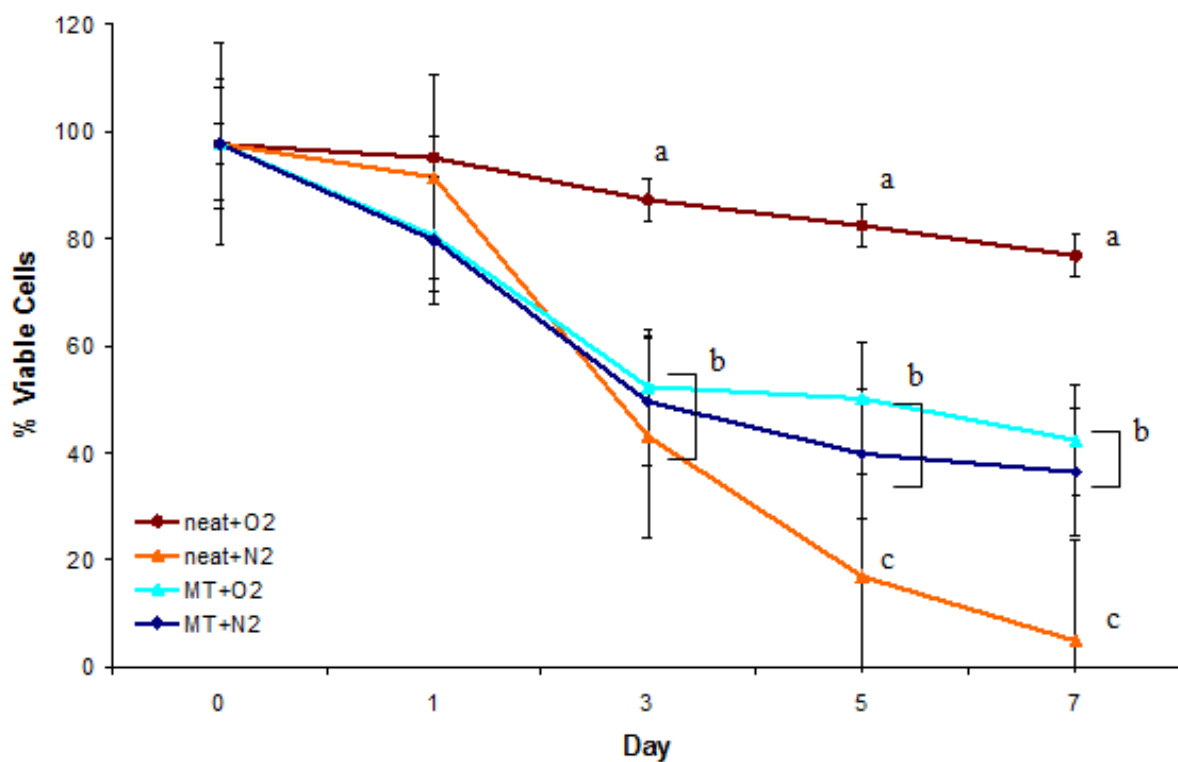


Figure 9. Viability of captive Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on Motility of Captive Atlantic Sturgeon Spermatozoa

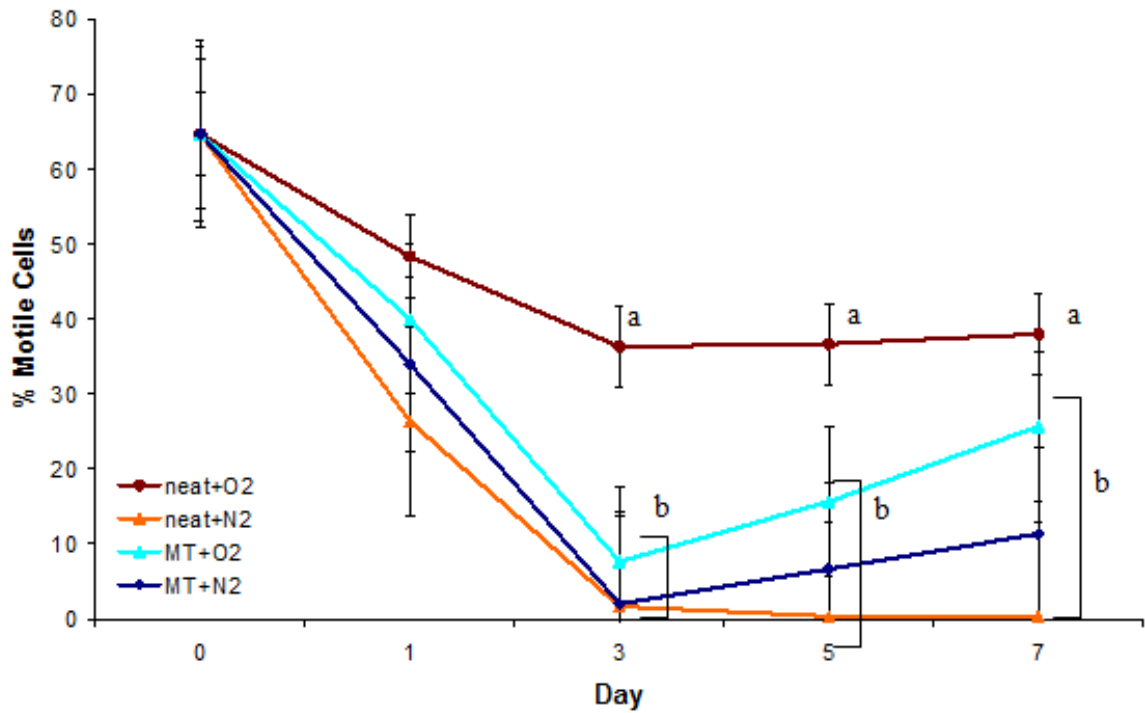


Figure 10. Motility of captive Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on Curvilinear Velocity of Captive Atlantic Sturgeon Spermatozoa

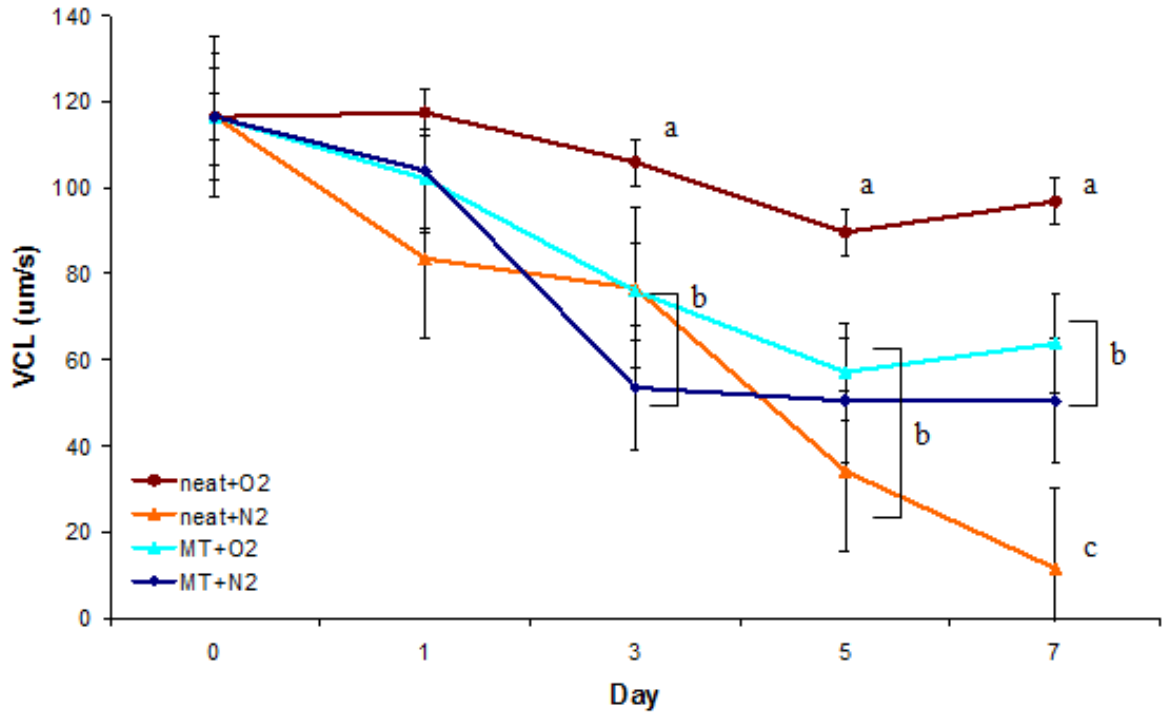


Figure 11. Curvilinear velocity of captive Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

Effect of O₂ vs. N₂ and Diluent on ATP Content of Captive Atlantic Sturgeon Spermatozoa

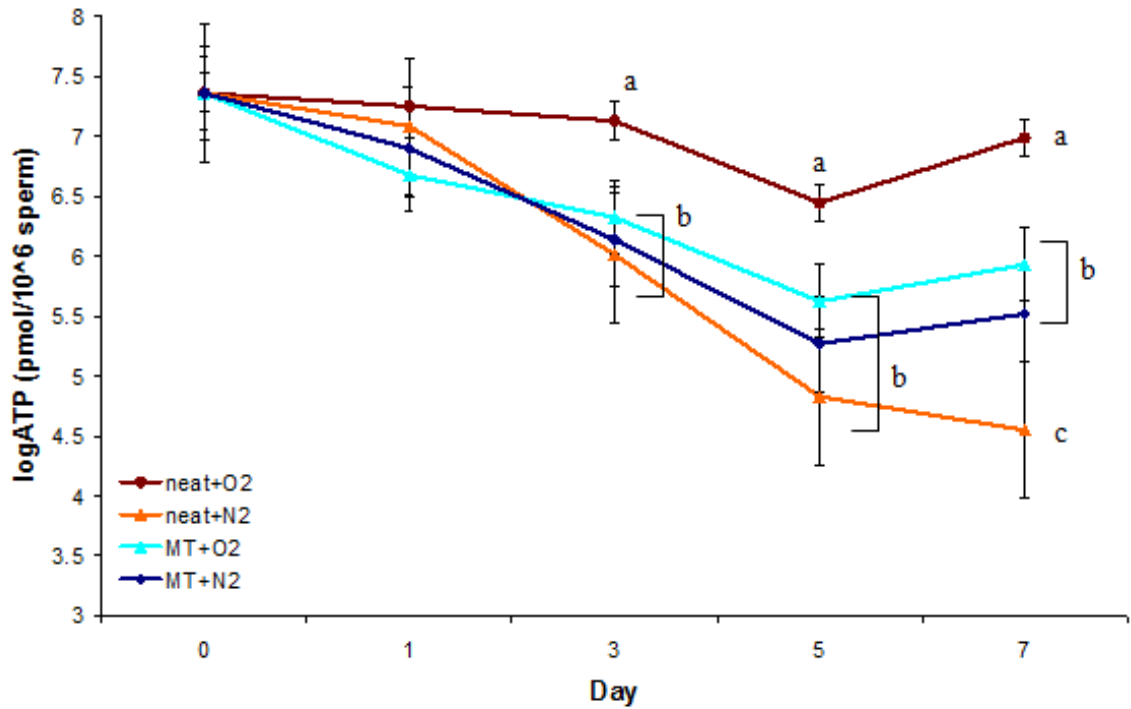


Figure 12. ATP content of captive Atlantic sturgeon sperm. Means (\pm SEM) with different subscripts are significantly different.

ANTIBIOTIC TRIAL

We tested the effects of antibiotics (pen-strep) on Atlantic sturgeon semen over a period of 21 days, hoping to control the growth of bacteria. Data were tested for normality of residuals, and all data were found to be normal. Viability and motility of wild Atlantic sturgeon spermatozoa stored with and without antibiotics over 21 days are shown in Figures 13 and 14, respectively. Results showed there was no significant improvement in cell quality associated with antibiotics. However, diluting resulted in a significantly higher percentage of viable ($P=0.0336$) cells and motile ($P=0.0082$) cells. Also, there was a significant ($P<0.0001$) time effect on the percentage of viable and motile cells.

The results from this very preliminary study, did suggest that sperm quality, in terms of viability and motility, could be significantly improved if samples were diluted rather than leaving the samples neat.

Viability in Atlantic Sturgeon Spermatozoa Treated with Antibiotics

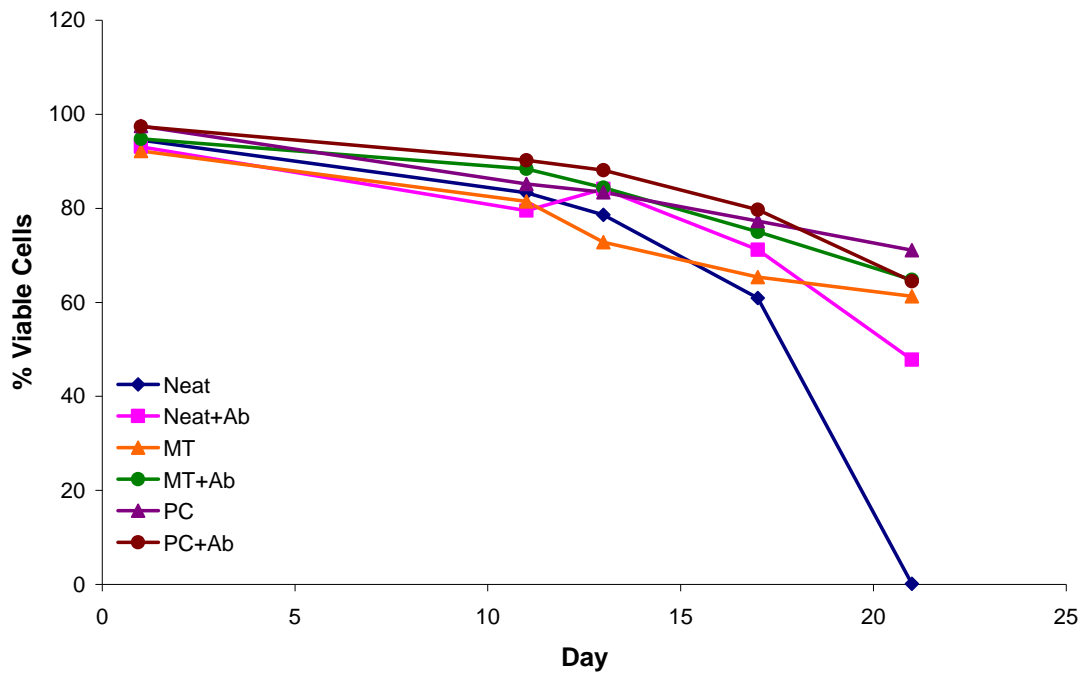


Figure 13. Viability of wild Atlantic sturgeon sperm treated with antibiotics. Day ($P < 0.0001$) and diluent ($P < 0.0336$) were statistically significant.

Motility in Atlantic Sturgeon Spermatozoa Treated with Antibiotics

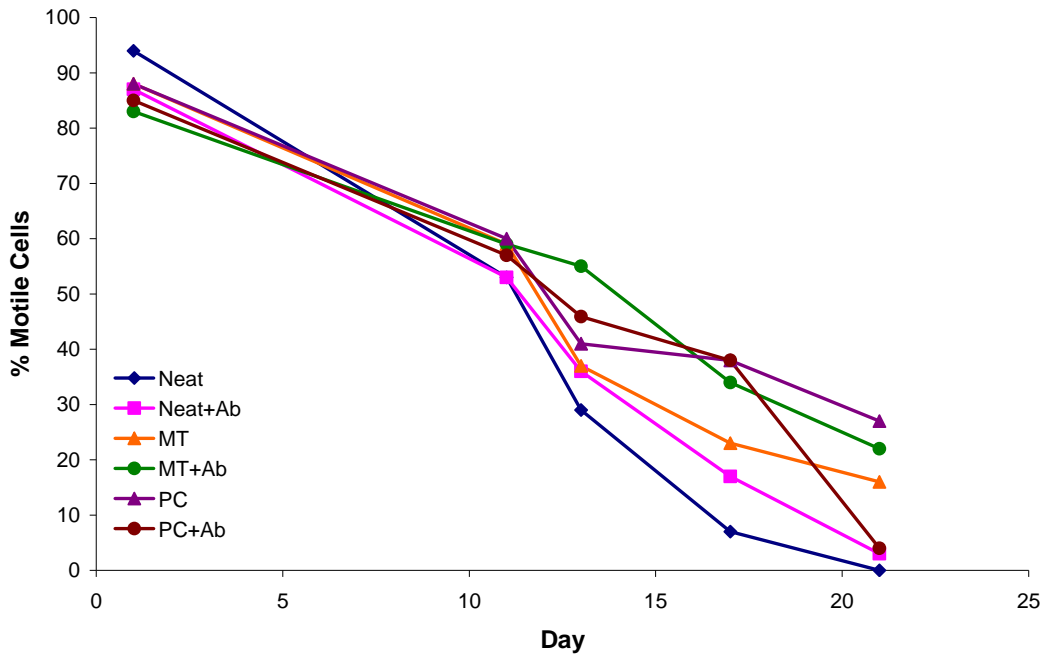


Figure 14. Motility of wild Atlantic sturgeon sperm treated with antibiotics. Day ($P < 0.0001$) and diluent ($P < 0.0082$) were statistically significant.

RESULTS: YEAR TWO

DAY 0 QUALITY ASSESSMENT

Table 3 (see Appendix) summarizes day 0 captive and wild samples obtained in the 2009 spawning season. The table provides information on initial sperm motility, pH, cell density and osmolality. Osmolalities between individual fish varied, both captive and wild, and ranged from 87 to 167 mOsm. The pH also varied and ranged from 7.2 to 7.8. Osmolalities of wild semen samples varied from 87-167mOsm (mean = 123.3mOsm), motilities varied from 10-95% (mean = 63.8%) and the pH varied from 7.2-7.7 (mean = 7.5). Osmolalities of captive semen samples ranged from 95-108mOsm (mean = 104.2mOsm), motilities varied from 0-60% (mean = 34%) and the pH ranged from 7.3-7.7 (mean = 7.5).

SHORT-TERM STORAGE STUDY

All data were not normal, with the exception of the wild fish motility data, thus were logarithmically transformed. Where there was a significant interaction, main effects were ignored, and simple effects were discussed.

WILD STURGEON

Figure 15 shows the differences in viability among the six treatments applied to wild Atlantic sturgeon sperm. There was a significant diluent ($P=0.0028$) and day ($P<0.0001$) effect on the viability of wild Atlantic sturgeon spermatozoa (Figure 16). At the end of the 21 day storage period, viability of samples diluted with the PC extender upon arrival to the lab was significantly higher than diluting at the time of collection or leaving the samples neat. However, viability of samples diluted at the time of collection was still higher than when samples were not diluted.

Figure 17 shows the differences in motility among the six treatments applied to wild Atlantic sturgeon sperm. There was a significant diluent ($P<0.0001$), gas ($P=0.0049$) and day ($P<0.0001$) effect on the motility of wild Atlantic sturgeon spermatozoa (Figures 18 & 19). At the end of the 21 day storage period, the percentage of motile cells was higher when samples were diluted with the PC extender upon arrival to the (PCL) compared to diluting at the time of collection or leaving the samples neat. However, motility of samples diluted at the time of collection (PCB) was still significantly higher leaving samples neat. In addition, by day 21, motility was highest when samples were stored in the presence of oxygen compared to samples stored under air.

Figure 20 shows the differences in VCL among the six treatments applied to wild Atlantic sturgeon sperm. There was a significant day by gas interaction ($P=0.0005$) on the curvilinear velocity of wild Atlantic sturgeon spermatozoa (Figure 21). At the end of the 21 day storage period, sperm cells were swimming fastest of samples stored in the presence of oxygen was significantly better than samples stored under air.

Figure 22 shows the differences in ATP content among the six treatments applied to wild Atlantic sturgeon sperm. There was a significant diluent ($P < 0.0001$) and day ($P < 0.0001$) effect on the energetics of wild Atlantic sturgeon spermatozoa (Figure 23). At the end of the 21 day storage period, ATP levels of samples diluted with the PC extender at time of collection (PCB) were significantly higher than when samples were diluted upon arrival to the lab (PCL), or leaving the samples neat. However, ATP levels of samples diluted upon arrival to the lab (PCL) were still significantly higher than when samples were not diluted at all (neat).

Results from the wild fish indicated that over a 21 day storage period, sperm quality remained highest when semen was diluted with the PC extender compared to undiluted samples. Furthermore, in terms of motility and curvilinear velocity, sperm quality was significantly higher when stored in the presence of oxygen compared to samples stored under air.

Effect of Gas and Diluent on Viability of Wild Atlantic sturgeon Spermatozoa

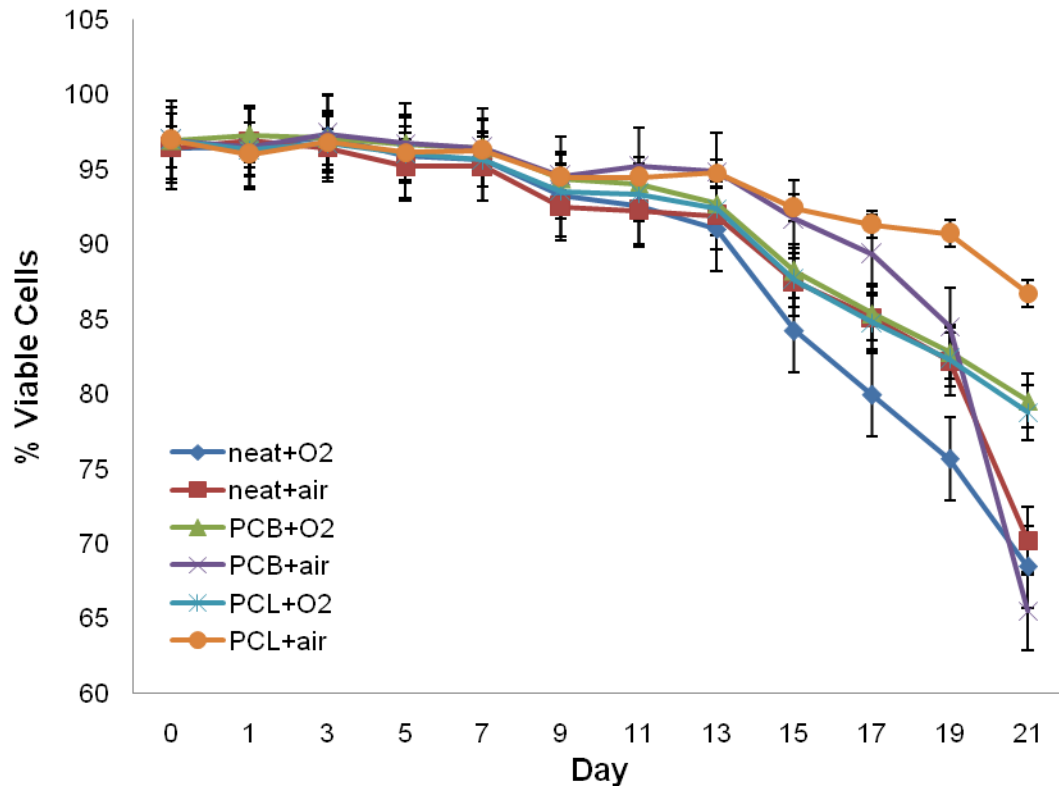


Figure 15. Viability (means \pm SEM) of wild Atlantic sturgeon sperm across 21 days.

Effect of Diluent on Viability of Wild Atlantic sturgeon Spermatozoa

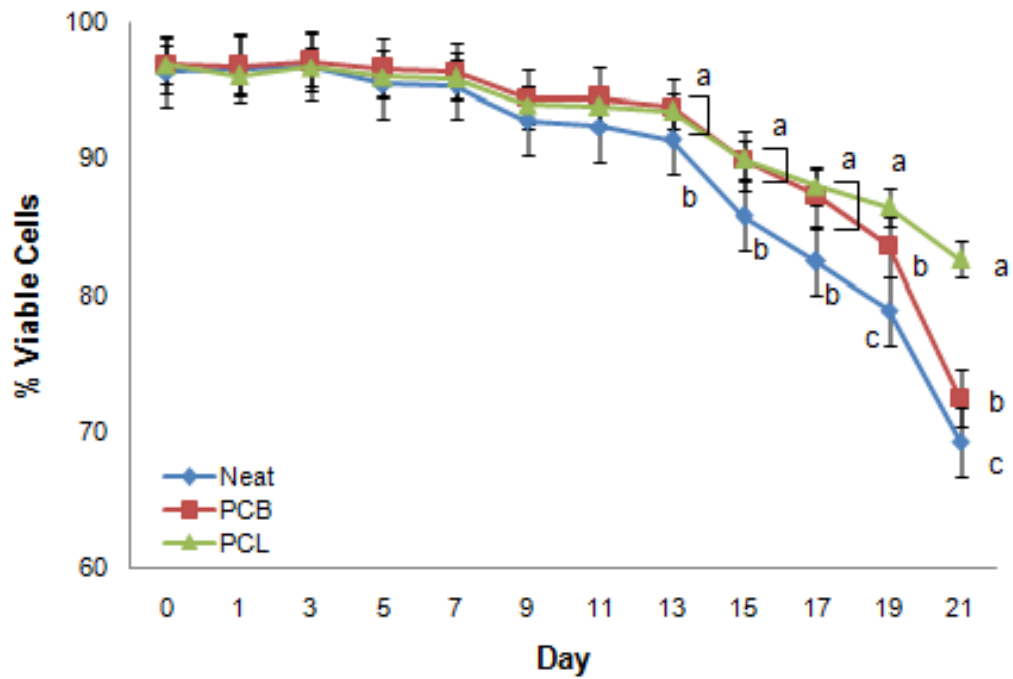


Figure 16. Diluent ($P=0.0028$) and day ($P<0.0001$) had a significant effect on viability of wild Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on Motility of Wild Atlantic sturgeon Spermatozoa

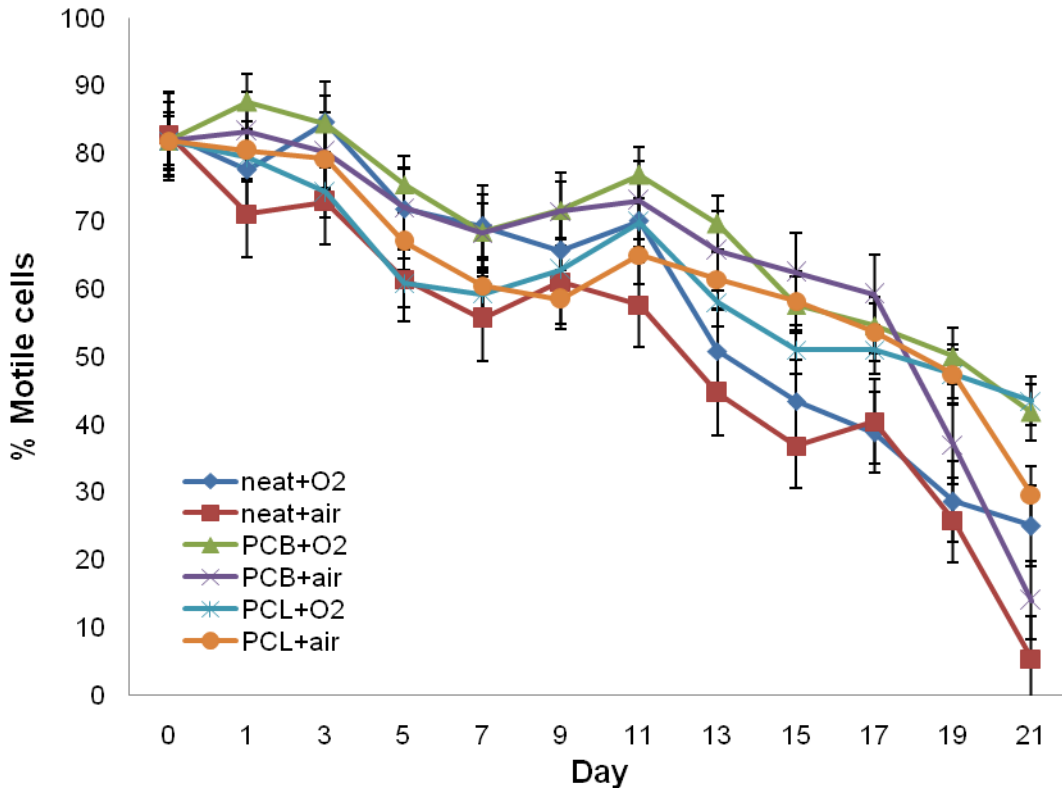


Figure 17. Motility (means± SEM) of wild Atlantic sturgeon sperm across 21 days.

Effect of Diluent on Motility of Wild Atlantic sturgeon Spermatozoa

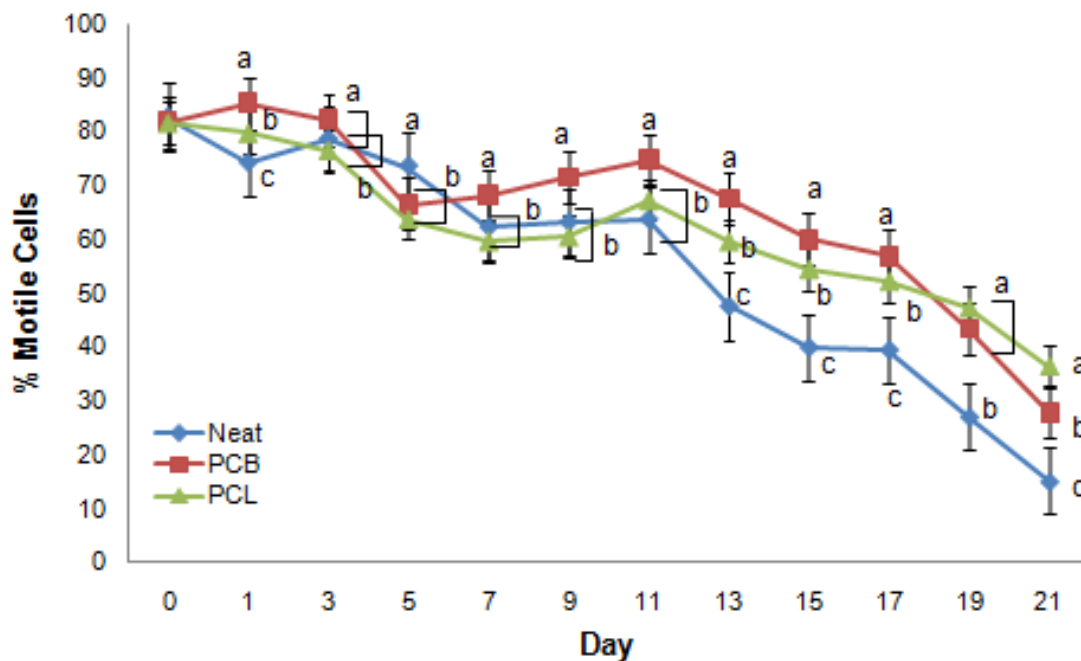


Figure 18. Diluent ($P < 0.0001$) and day ($P < 0.0001$) had a significant effect on motility of wild Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas on Motility of Wild Atlantic sturgeon Spermatozoa

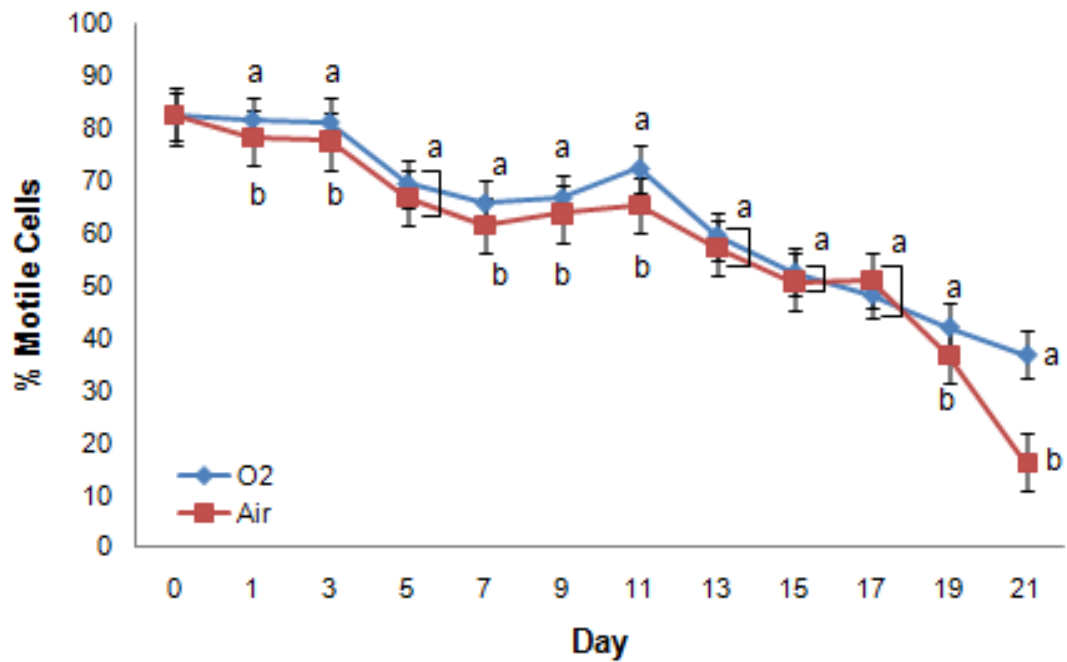


Figure 19. Gas ($P=0.0049$) and day ($P<0.0001$) had a significant effect on motility of wild Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on Curvilinear Velocity of Wild Atlantic sturgeon Spermatozoa

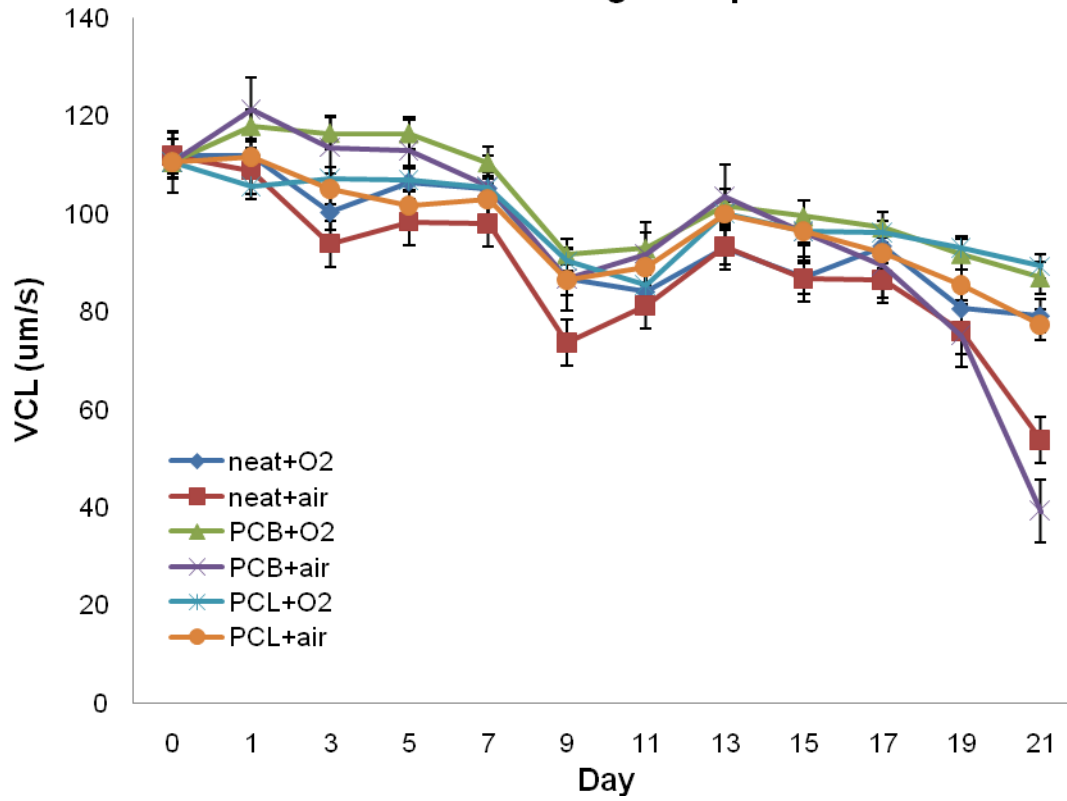


Figure 20. Curvilinear velocity (means \pm SEM) of wild Atlantic sturgeon sperm across 21 days.

Effect of Gas on Curvilinear Velocity of Wild Atlantic sturgeon Spermatozoa

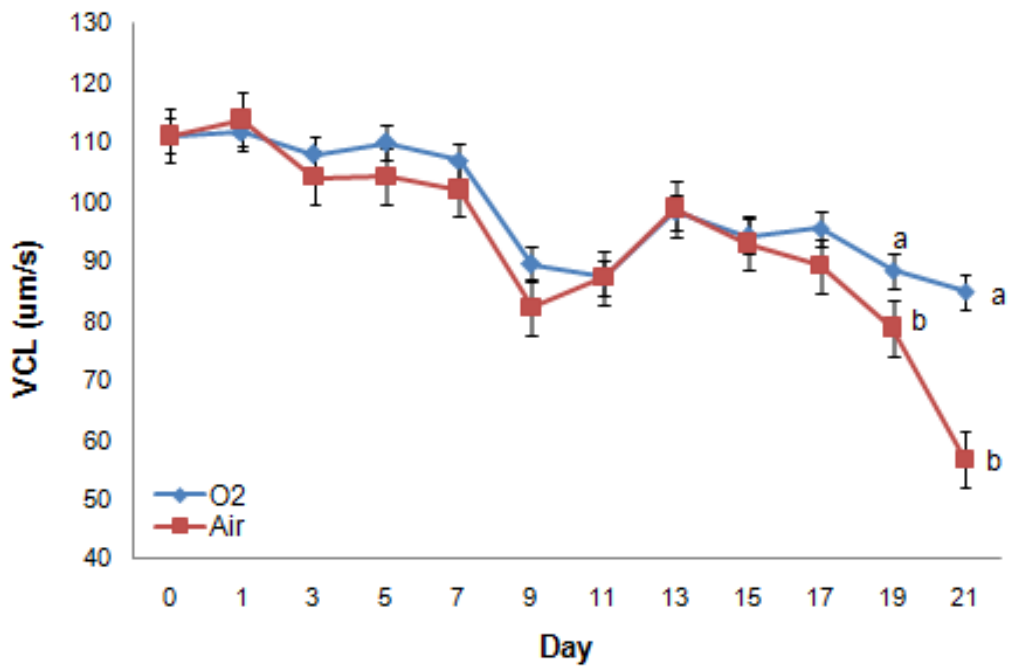


Figure 21. There was a significant day by gas interaction ($P=0.0005$) effect on the curvilinear velocity of wild Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on ATP Levels in Wild Atlantic sturgeon Spermatozoa

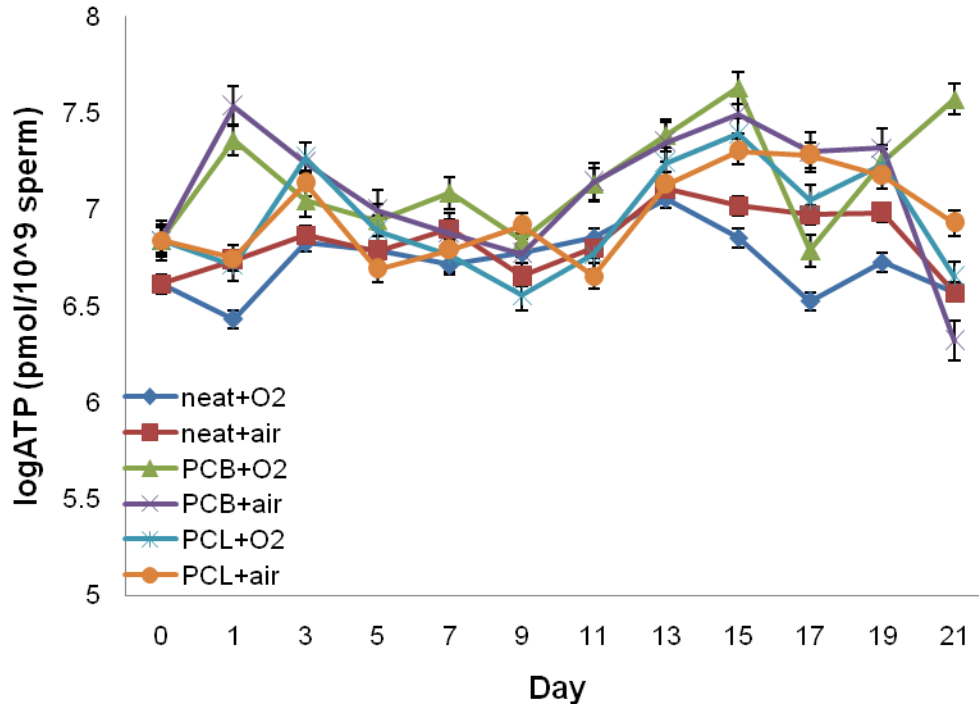


Figure 22. ATP content (means \pm SEM) of wild Atlantic sturgeon sperm across 21 days.

Effect of Diluent on ATP Content of Wild Atlantic sturgeon Spermatozoa

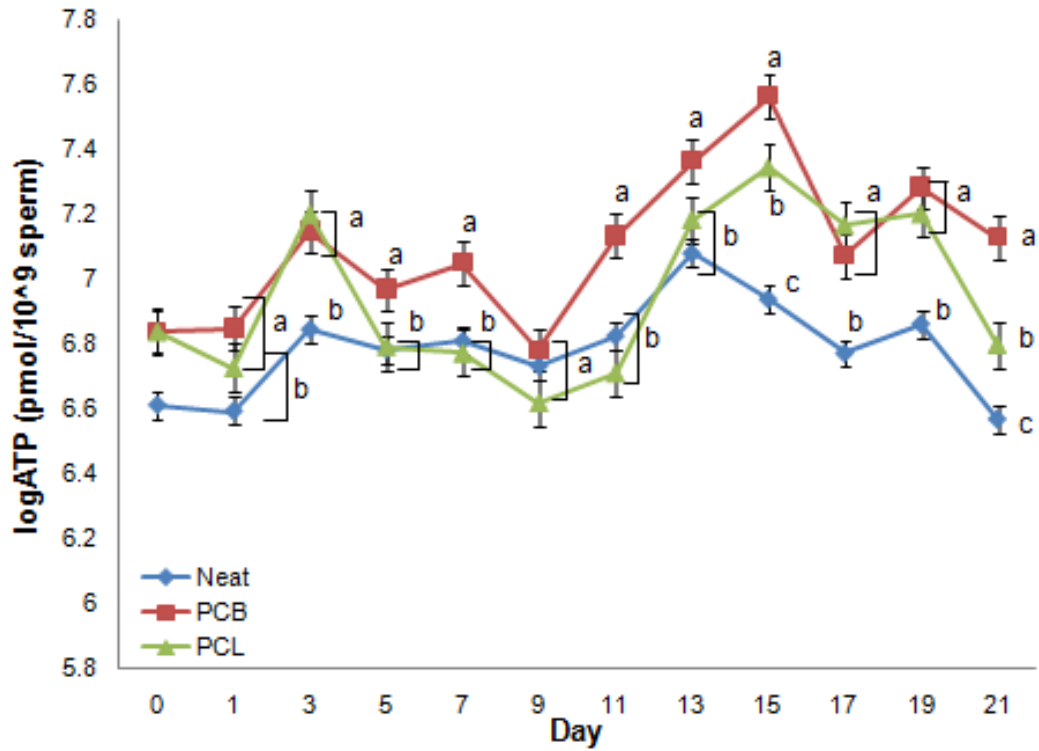


Figure 23. Diluent ($P < 0.0001$) and day ($P < 0.0001$) had a significant effect on the ATP content of wild Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

CAPTIVE STURGEON

Figure 24 represents the differences in viability among the six treatments applied to captive Atlantic sturgeon sperm. There was a significant gas ($P=0.0039$) and day ($P<0.0001$) effect on the viability of captive Atlantic sturgeon spermatozoa (Figure 25). Captive samples did not survive through the entire 21 day storage period. By day 19, all samples were non-viable. However, at the end of 15 days, viability of samples treated with oxygen was significantly higher than samples treated with air.

Figure 26 shows the differences in motility among the six treatments applied to captive Atlantic sturgeon sperm across. There was a significant day by diluent interaction ($P=0.003$) on motility of captive Atlantic sturgeon spermatozoa (Figure 27). The beneficial effect on gamete quality of diluting samples decreased as time progressed. Captive samples did not remain motile throughout the entire 21 day storage period. By day 13, all samples were non-motile.

Differences in VCL among the six treatments applied to captive Atlantic sturgeon spermatozoa can be seen in Figure 28. There was a significant day by diluent interaction ($P=0.0465$) on curvilinear velocity of captive Atlantic sturgeon spermatozoa (Figure 27). The beneficial effect on gamete quality of diluting samples decreased as time progressed.

Figure 30 illustrates the differences in ATP content among the six treatments applied to wild Atlantic sturgeon sperm. There was a significant diluent ($P=0.0007$) and day ($P<0.0001$) effect on the ATP content of captive Atlantic sturgeon spermatozoa (Figure 31). Captive samples did not last through the entire twenty-one day storage period. By day 11, all cells had been exhausted of their energy. However, at the end of seven days, cellular energy remained highest when samples were diluted with the PC

extender upon arrival to the lab (PCL). But, ATP levels were still significantly higher when samples were diluted with the PC extender at time of collection (PCB) than when samples were not diluted.

Results from captive fish indicated that sperm viability was significantly higher during storage, when samples were maintained in the presence of oxygen compared to those stored under air. ATP levels remained higher if samples were diluted with the PC extender upon arrival to the lab. Diluting captive sturgeon semen samples had a positive, significant effect on motility and curvilinear velocity; however, this positive effect decreased as time progressed.

Effect of Gas and Diluent on Viability of Captive Atlantic sturgeon Spermatozoa

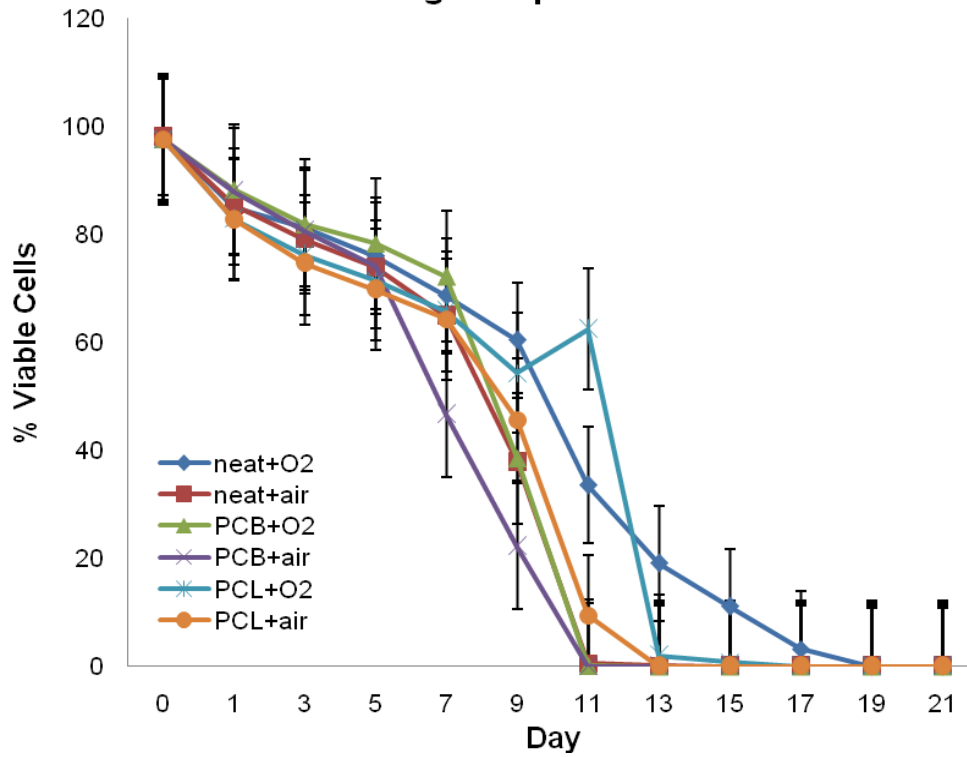


Figure 24. Viability (means \pm SEM) of captive Atlantic sturgeon sperm across 21 days.

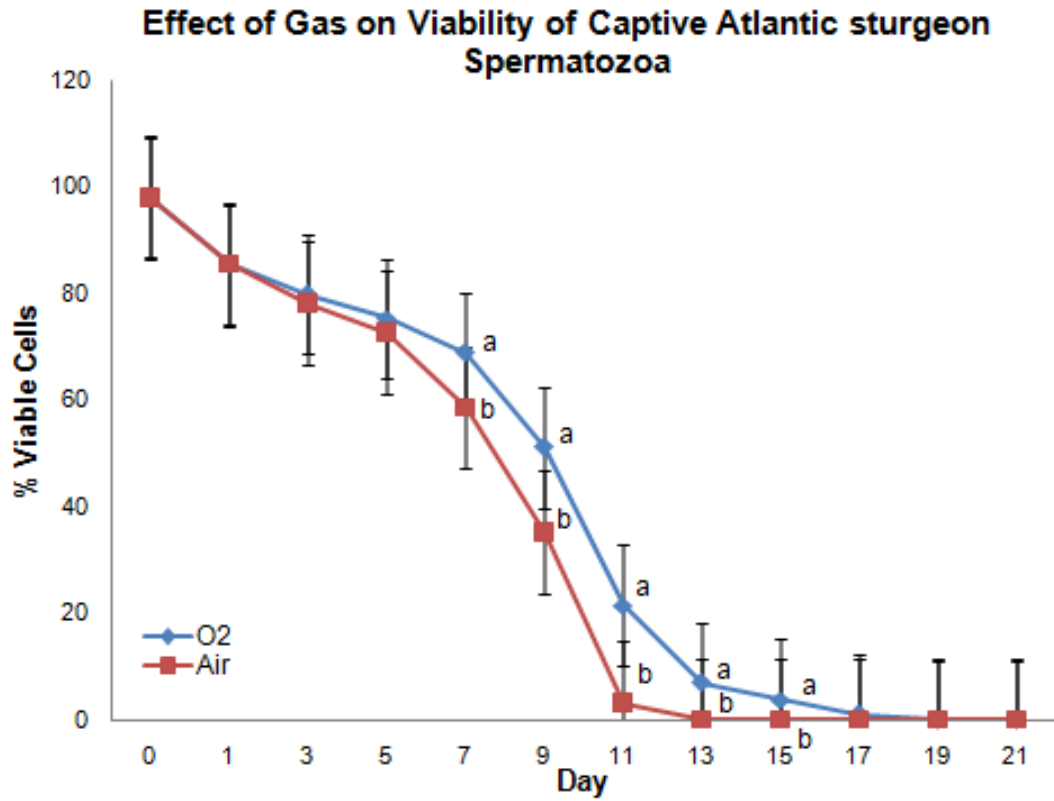


Figure 25. Gas ($P=0.0039$) and day ($P<0.0001$) had a significant effect on viability of captive Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on Motility of Captive Atlantic sturgeon Spermatozoa

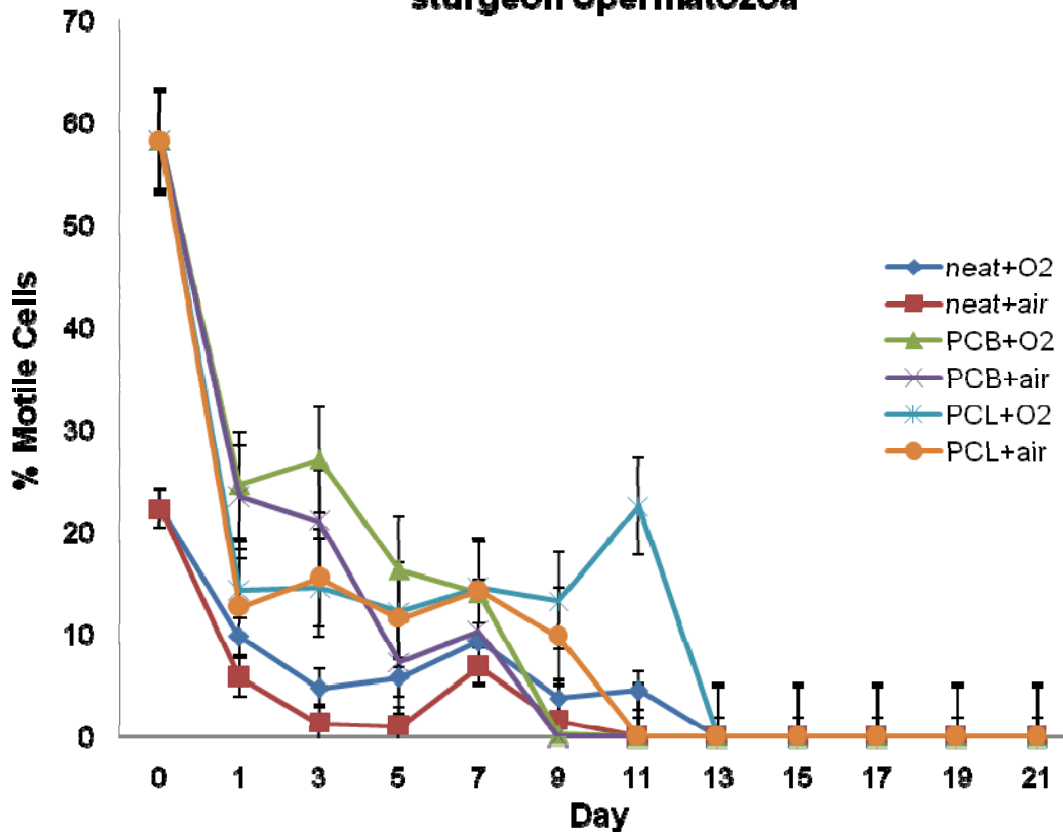


Figure 26. Motility (means± SEM) of captive Atlantic sturgeon sperm across 21 days.

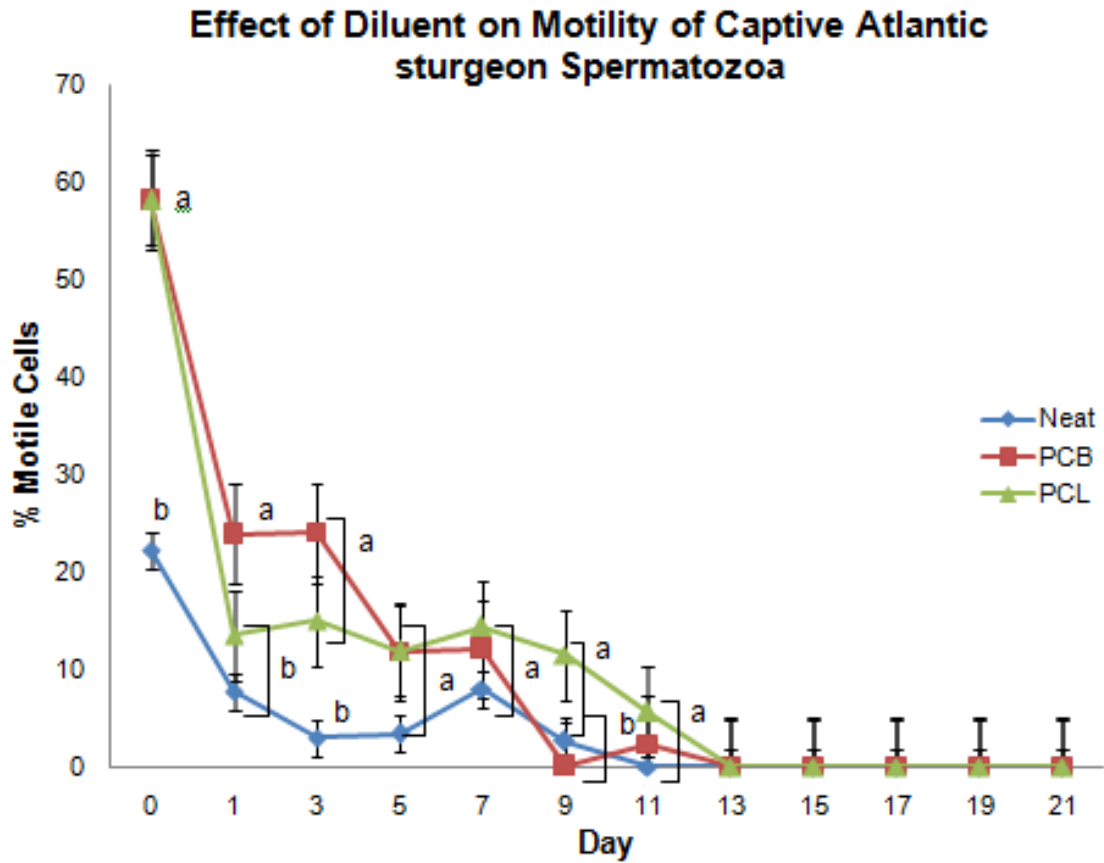


Figure 27. There was a significant day by diluent ($P=0.003$) interaction effect on motility of captive Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on Curvilinear Velocity of Captive Atlantic sturgeon Spermatozoa

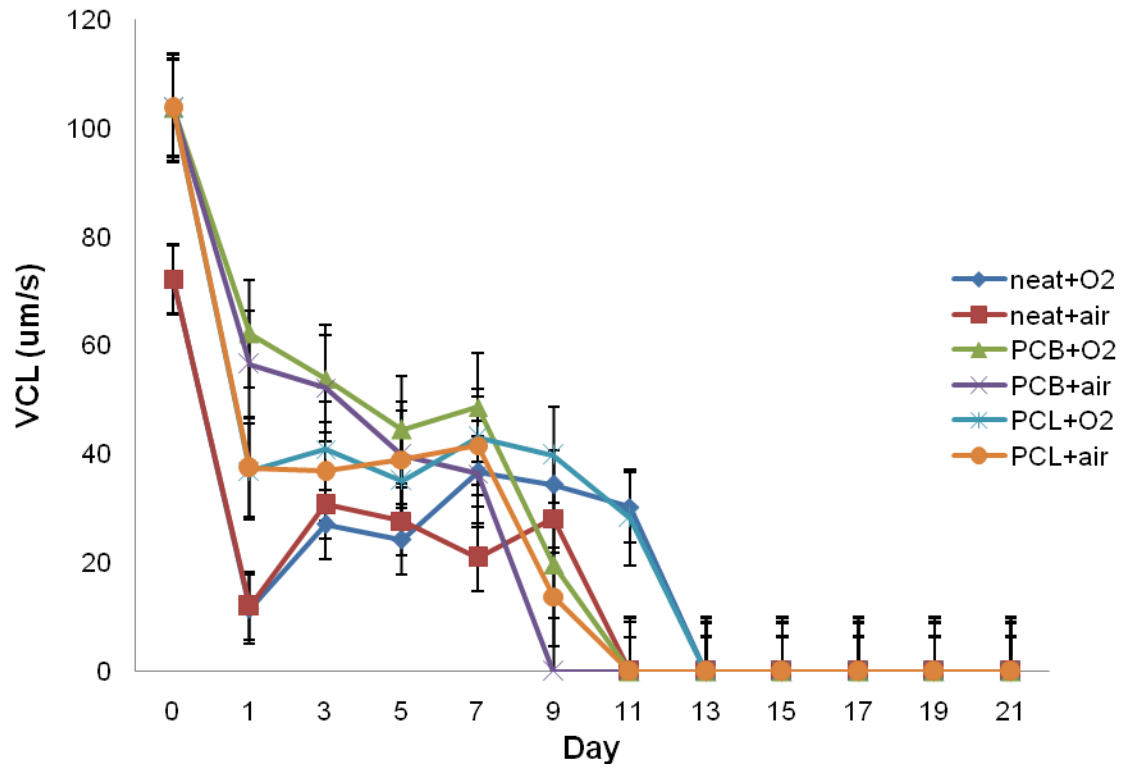


Figure 28. Curvilinear velocity (means \pm SEM) of captive Atlantic sturgeon sperm across 21 days.

Effect of Diluent on Curvilinear Velocity of Captive Atlantic sturgeon Spermatozoa

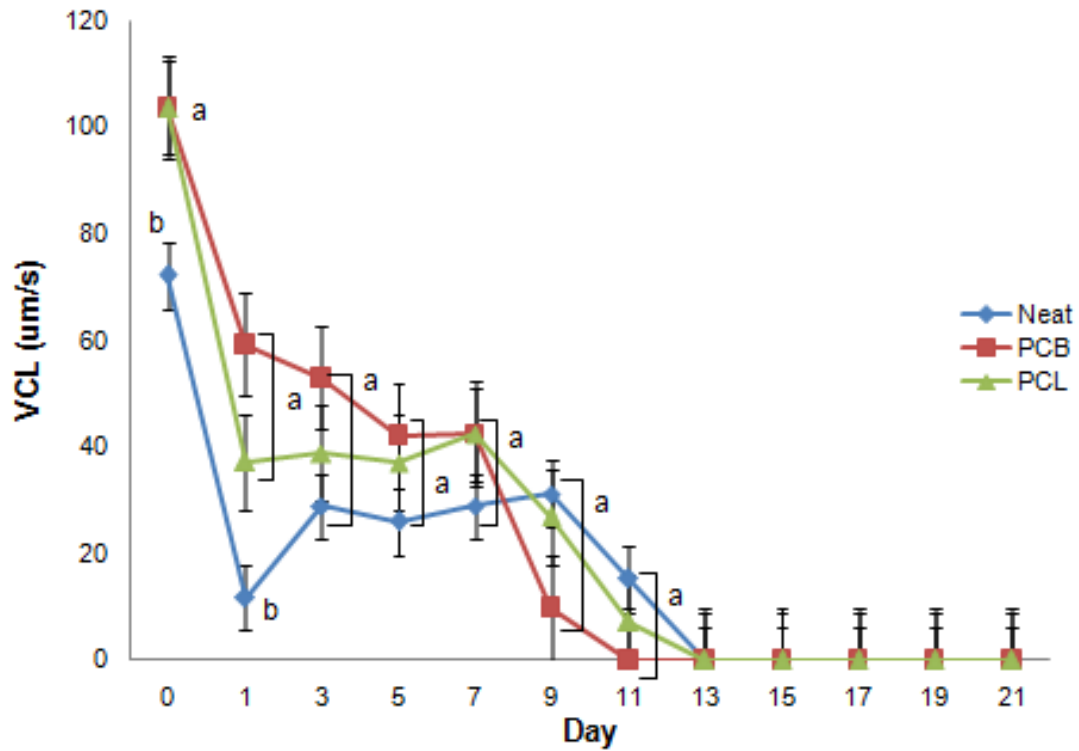


Figure 29. There was a significant day by diluent interaction ($P=0.0465$) effect on the curvilinear velocity of captive Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

Effect of Gas and Diluent on ATP Content of Captive Atlantic sturgeon Spermatozoa

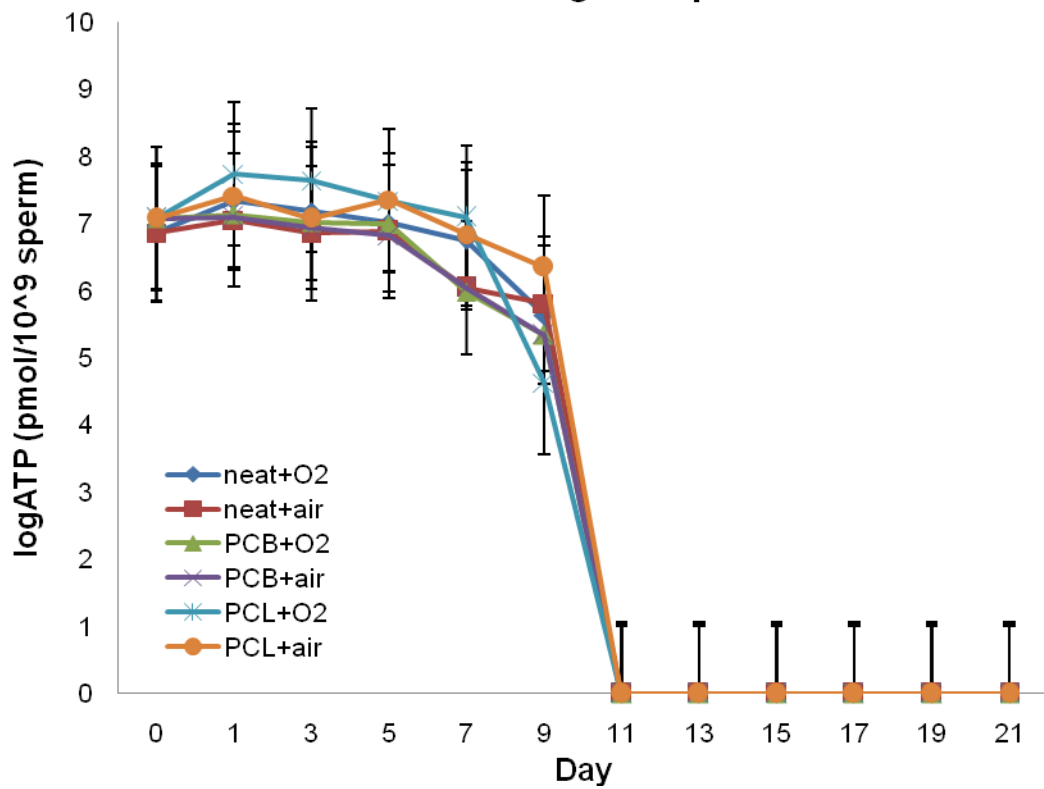


Figure 30. ATP content (means \pm SEM) of captive Atlantic sturgeon sperm across 21 days.

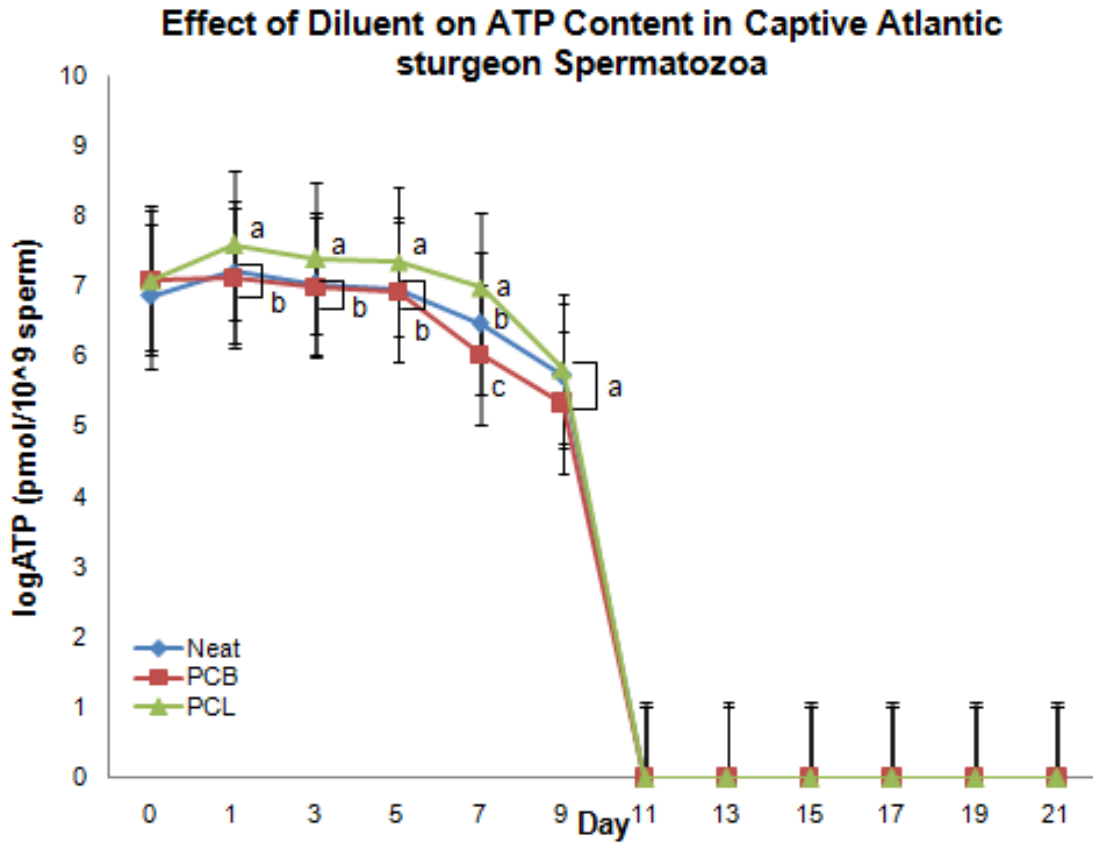


Figure 31. Diluent ($P=0.0007$) and day ($P<0.0001$) had a significant effect on the ATP content of captive Atlantic sturgeon spermatozoa across 21 days. Means (\pm SEM) with different subscripts are significantly different.

CONCLUSIONS

It was 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. In fact, year one's results revealed that spermatozoa stored with oxygen for seven days were of higher quality, in terms of viability, motility, curvilinear velocity and cell ATP level, than those stored in the absence of oxygen.

The results from the first year's study suggesting the importance of dilution and oxygen treatments were confirmed by year two's research over an expanded storage period. These results provide the basis for an effective successful short-term storage protocol for Atlantic sturgeon semen. Based on the results, Atlantic sturgeon sperm should be diluted with the PC extender and stored in the presence of oxygen.

Statistically it cannot be suggested that diluting immediately upon collection is the best dilution method, because results were inconsistent across the four variables measured. In terms of viability and motility, diluting one day post collection (PCL) resulted in significantly higher sperm quality. However, diluting immediately upon collection (PCB) resulted in significantly higher cellular ATP levels. But, biologically it seems that diluting immediately upon collection would be most beneficial, because the extender may mitigate the effects of any potential contamination from water or urine.

Because the Gulf sturgeon (*Acipenser oxyrinchus desotoi*) and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) are closely related subspecies of the same genus, the positive, significant effects of using the PC extender may be due to the fact that they share similar seminal plasma characteristics. Park & Chapman (2005) measured

concentrations of sodium chloride, glucose and potassium in the seminal plasma of the Gulf sturgeon and developed their extender based on these measurements.

Although not statistically tested, there was an apparent difference between the quality of the captive and wild sturgeon samples upon arrival to our facility; the captive semen samples being of much poorer quality than those of the wild fish (see Figures 32-39 in Appendix). In fact in year one's study, three out of the seven captive semen samples collected lacked cells in the seminal plasma and thus were not used in the study. In addition, most of the captive samples that did possess sperm cells still had densities orders of magnitude less than those of the wild fish. The cause(s) for the observed decrease in semen quality of the captive sturgeon when contrasted with the wild sturgeon is unknown, but may be linked to differences associated with the environmental conditions, including nutrition for the captive populations. Stressors such as physical handling and living in a confined space may also have some effect on decreased sperm quality.

Sperm quality of captive samples decreased rapidly and in fact, spermatozoa from these samples did not survive through year two's 21 day storage experiment. A possible reason for the sudden drop in quality of captive semen samples could be the growth of bacterial populations coupled with the already lower initial sperm quality, compared to wild samples. Thus, as a next step the effects of different antibiotics to control the growth of bacterial populations should be studied more in depth. This could prove most beneficial for captive samples, since the data collected in the above studies shows samples maintaining quality only for about one week.

The results of these studies should allow for better management of increasingly scarce Atlantic sturgeon gametes. It is anticipated that with efficacious techniques, biologists will not only be better supplied with gametes necessary to generate new recruits for shrinking Atlantic sturgeon populations whenever limited opportunities arise, but will also be able to conserve important genetic variation obtained from remaining Atlantic sturgeon populations. It is hoped that these results may also prove useful as initial cryopreservation protocols for the Atlantic sturgeon are developed.

APPENDIX

Table 1. Recipes for the Park & Chapman, Modified Tsvetkova and Tris solutions.

| Solution | pH | Osmolality | Contents |
|--|-----------|------------|---|
| Park & Chapman (Park & Chapman, 2005) | 7.3 - 7.5 | 80 - 100 | To 1L of distilled water, add: 1.0g sodium chloride 0.2g potassium chloride 0.5g sodium bicarbonate 0.05g calcium chloride (anhydrous) 0.05g magnesium sulfate 0.15g sodium phosphate monobasic 0.15g sodium phosphate dibasic (anhydrous) |
| Modified Tsvetkova (Horvath et al., 2005) | 8.0 | 80 | To 985mL of distilled water, add: 23.4mM (8.01g) sucrose 30mM (3.63g) Tris 0.25mM (0.07g) KCl |
| Tris | 8.0 | 20 | To 1L distilled water, add: 20mM (2.42g) Tris |

Table 2. Summary of captive and wild caught Atlantic sturgeon Day 0 semen samples (2008).

| CAPTIVE FISH | | | | | | |
|---------------------|---------------|-----------------------|---------------------------|-----------|--------------------------|---------------------|
| Collected | Fish # | 2008 AS P.I.T. | Density (cells/mL) | pH | Osmolality (mOsm) | Motility (%) |
| 6.09.08 | 1 | 9270F | 4.1 X 10 ⁸ | 8.3 | 77 | 60 |
| 6.09.08 | 2 | 86A2C | 4.8 X 10 ⁷ | 8.4 | 66 | 95 |
| 6.09.08 | - | 32857 | 0 cells | - | - | - |
| 6.09.08 | - | E326F | 0 cells | - | - | - |
| 6.10.08 | - | 1294F | 0 cells | - | - | - |
| 6.10.08 | - | E1A03 | 7.4 X 10 ⁹ | 7.4 | 113 | 0 |
| 6.10.08 | 3 | E3B2D | 3.7 X 10 ⁹ | 7.4 | 74 | 90 |

| WILD FISH | | | | | | |
|------------------|---------------|-----------------------|---------------------------|-----------|--------------------------|---------------------|
| Collected | Fish # | 2008 AS P.I.T. | Density (cells/mL) | pH | Osmolality (mOsm) | Motility (%) |
| 6.17.08 | Old 4 | 5FE80 | 4.2 X 10 ⁹ | 7.5 | 117 | 95 |
| 6.17.08 | Old 5 | 5FCF5 | 8.3 X 10 ⁹ | 7.3 | 106 | 50 |
| 6.17.08 | Old 6 | 6208F | 10.6 X 10 ⁹ | 7.2 | 85 | 50 |
| 6.19.08 | New 4 | 257C659613 | 2.2 X 10 ⁹ | 7.9 | 75 | 95 |
| 6.19.08 | New 5 | 257C63C87F | - | 7.3 | 150 | 70 |
| 6.19.08 | New 6 | 257C63DEF1 | 10.3 X 10 ⁹ | 7.6 | 91 | 95 |
| 6.19.08 | 7 | 257C63D664 | - | 7.3 | 150 | 60 |
| 6.19.08 | 8 | 467C333A47 | 13.4 X 10 ⁹ | 7.6 | 99 | 90 |
| 6.19.08 | 9 | 257C65653B | - | 7.5 | 150 | 60 |
| 6.19.08 | 10 | 257C64DB4D | 3.2 X 10 ⁹ | 7.6 | 97 | 95 |
| 6.19.08 | 11 | 257C65D5FC | 9.3 X 10 ⁹ | 7.6 | 90 | 90 |

Table 3. Summary of wild and captive caught Atlantic sturgeon Day 0 semen samples (2009).

| WILD FISH | | | | | | |
|------------------|---------------|---------------|---|-----------|--------------------------|---------------------|
| Collected | Fish # | P.I.T. | Density X10⁹ (cells/mL) | pH | Osmolality (mOsm) | Motility (%) |
| 6.16.09 | H09-01 | | 3.3 | 7.4 | 87 | 95 |
| 6.17.09 | H09-02 | | 2.3 | 7.8 | 89 | 92 |
| 6.17.09 | H09-03 | | 2.9 | 7.7 | 120 | 90 |
| 6.18.09 | H09-04 | | 9.7 | 7.2 | 104 | 83 |
| 6.18.09 | H09-05 | | 10.9 | 7.3 | 167 | 67 |
| 6.18.09 | H09-06 | | 2.2 | 7.5 | 133 | 10 |
| 6.18.09 | H09-07 | | 3.1 | 7.4 | 145 | 50 |
| 6.18.09 | H09-08 | | 2.7 | 7.4 | 142 | 43 |
| 6.18.09 | H09-09 | | 2.8 | 7.6 | 100 | 90 |
| 6.18.09 | H09-10 | | 4.0 | 7.5 | 134 | 25 |
| 6.18.09 | H09-11 | | 12.7 | 7.6 | 128 | 73 |
| 6.18.09 | H09-12 | | 1.9 | 7.6 | 130 | 47 |

| CAPTIVE FISH | | | | | | |
|---------------------|---------------|---------------|---|-----------|--------------------------|---------------------|
| Collected | Fish # | P.I.T. | Density X10⁹ (cells/mL) | pH | Osmolality (mOsm) | Motility (%) |
| 6.25.09 | L09-13 | 17B63 | 4.7 | 7.7 | 106 | 60 |
| 6.25.09 | L09-14 | 9270F | 16.4 | 7.3 | 104 | 0 |
| 6.25.09 | L09-15 | 85257 | 3.1 | 7.7 | 108 | 60 |
| 6.25.09 | L09-16 | E1A03 | 8.2 | 7.3 | 108 | 30 |
| 6.25.09 | L09-17 | 1294F | 3.5 | 7.6 | 95 | 20 |

Comparison of Viability In Captive and Wild Atlantic Sturgeon Spermatozoa Stored Neat+O₂

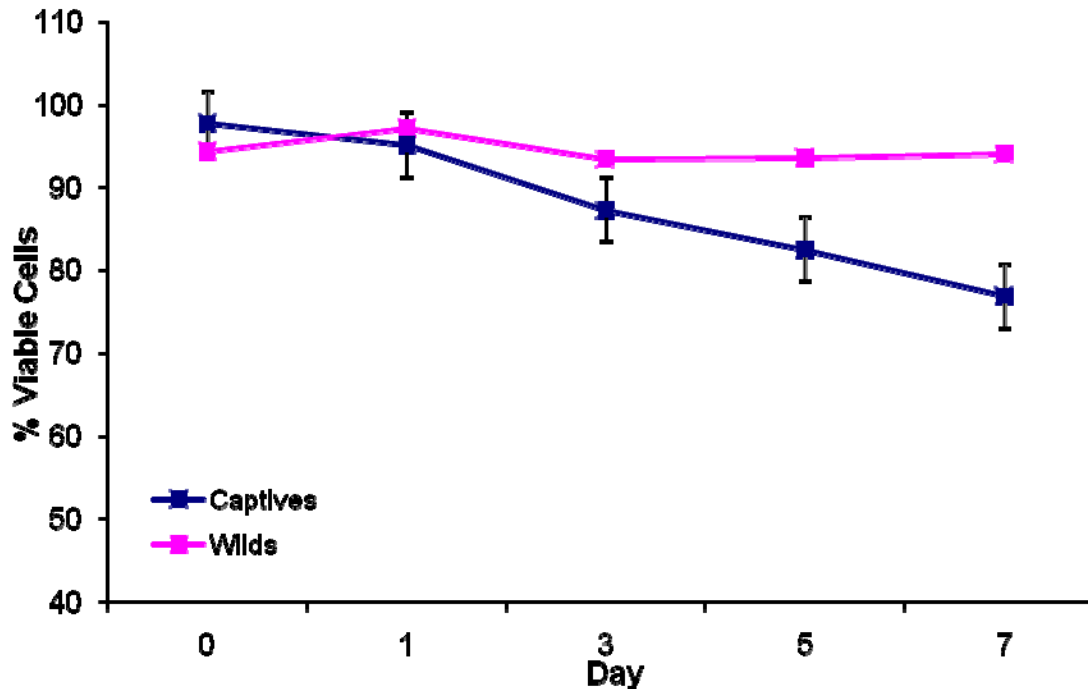


Figure 32. Differences in viability (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored undiluted for seven days with oxygen (2008).

Comparison of Motility In Captive and Wild Atlantic Sturgeon Spermatozoa Stored Neat+O₂

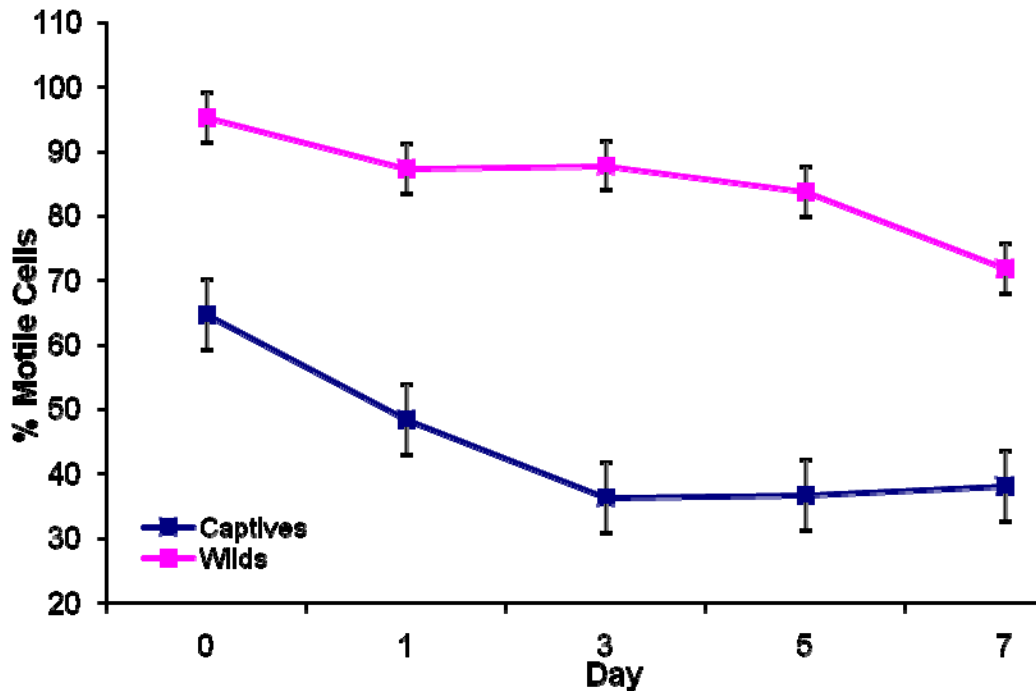


Figure 33. Differences in motility (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored undiluted for seven days with oxygen (2008).

Comparison of Curvilinear Velocity in Captive and Wild Atlantic Sturgeon Spermatozoa Stored Neat+O₂

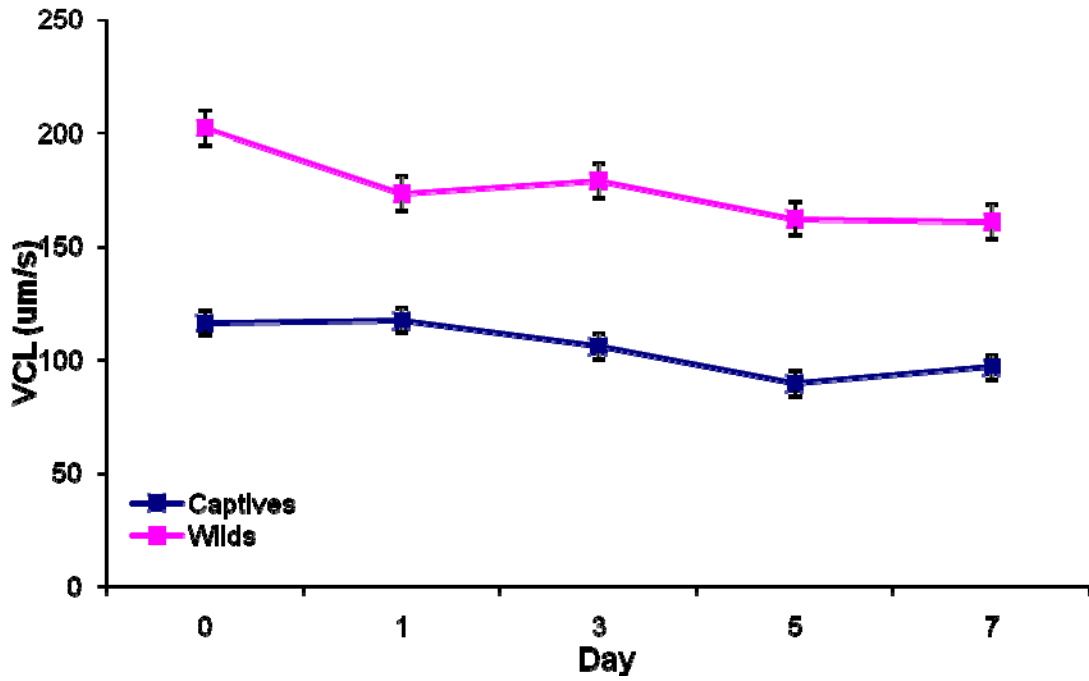


Figure 34. Differences in curvilinear velocity (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored undiluted for seven days with oxygen (2008).

Comparison of ATP Content In Captive and Wild Atlantic Sturgeon Spermatozoa Stored Neat+O₂

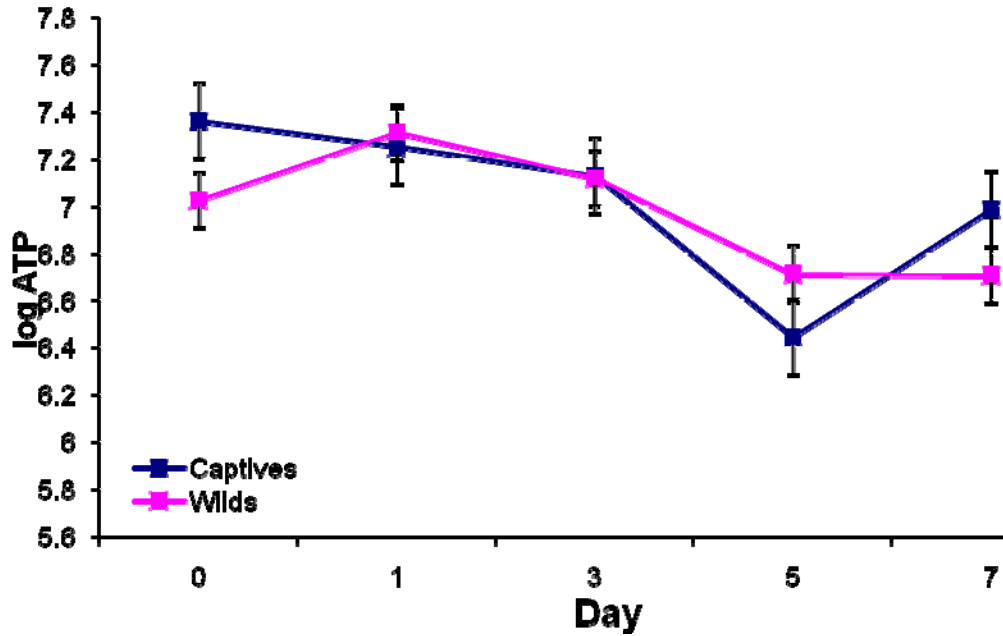


Figure 35. Differences in ATP content (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored undiluted for seven days with oxygen (2008).

Comparison of Viability in Captive and Wild Atlantic sturgeon Spermatozoa Stored PCB+O2

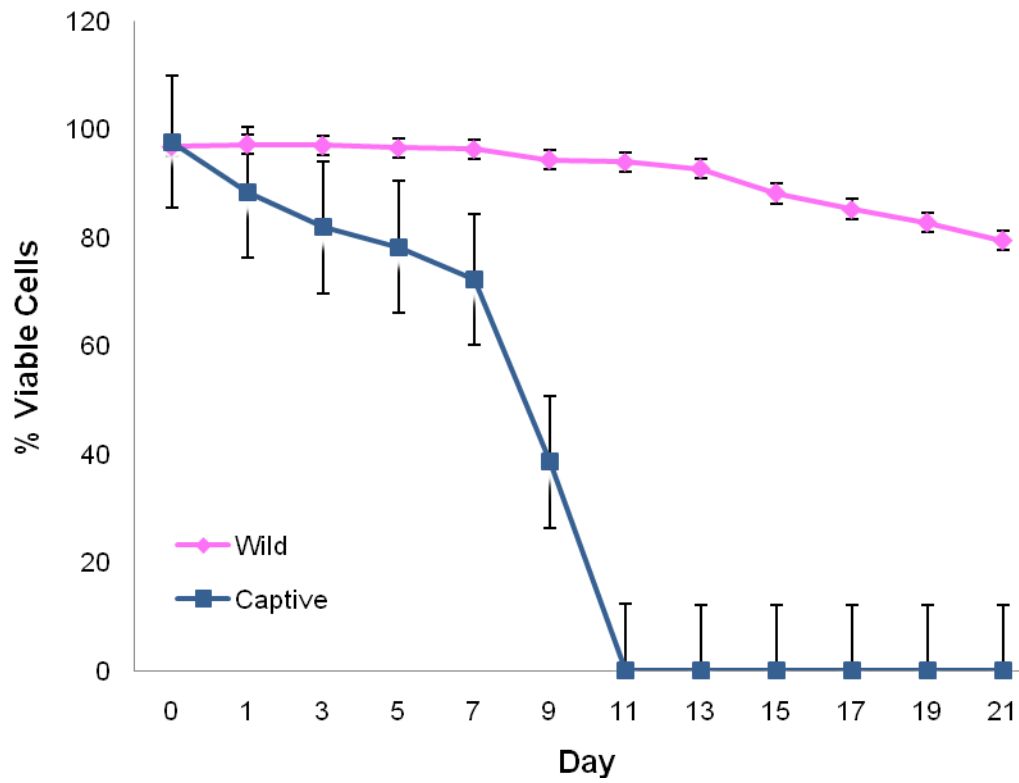


Figure 36. Differences in viability (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored diluted (PC extender) for 21 days with oxygen (2009).

Comparison of Motility in Captive and Wild Atlantic sturgeon Spermatozoa Stored PCB+O2

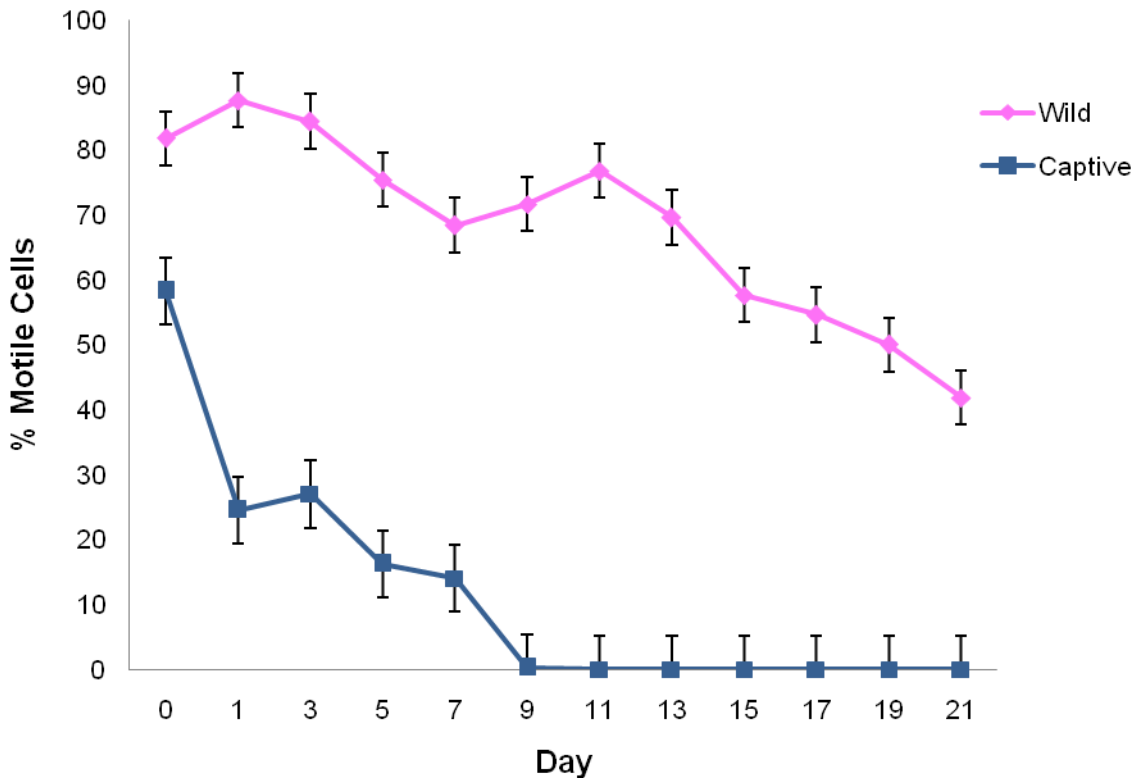


Figure 37. Differences in motility (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored diluted (PC extender) for 21 days with oxygen (2009).

Comparison of Curvilinear Velocity in Captive and Wild Atlantic sturgeon Spermatozoa Stored PCB+O2

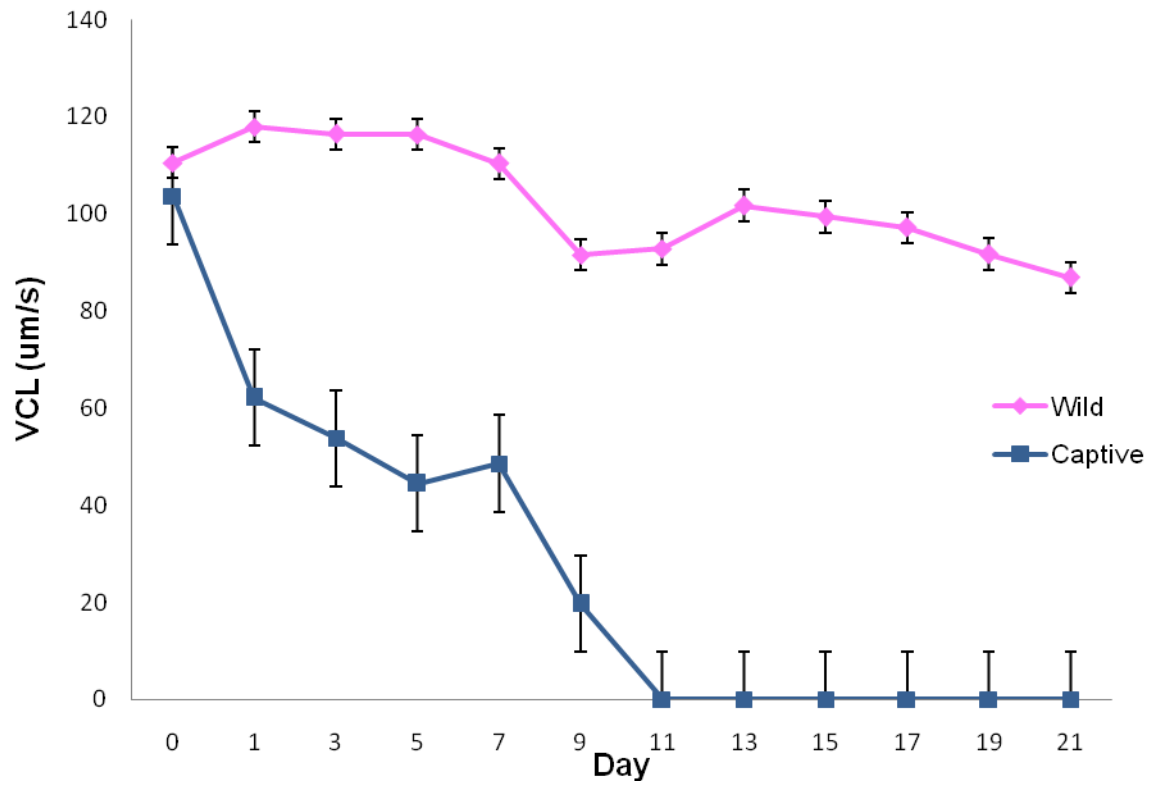


Figure 38. Differences in curvilinear velocity (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored diluted (PC extender) for 21 days with oxygen (2009).

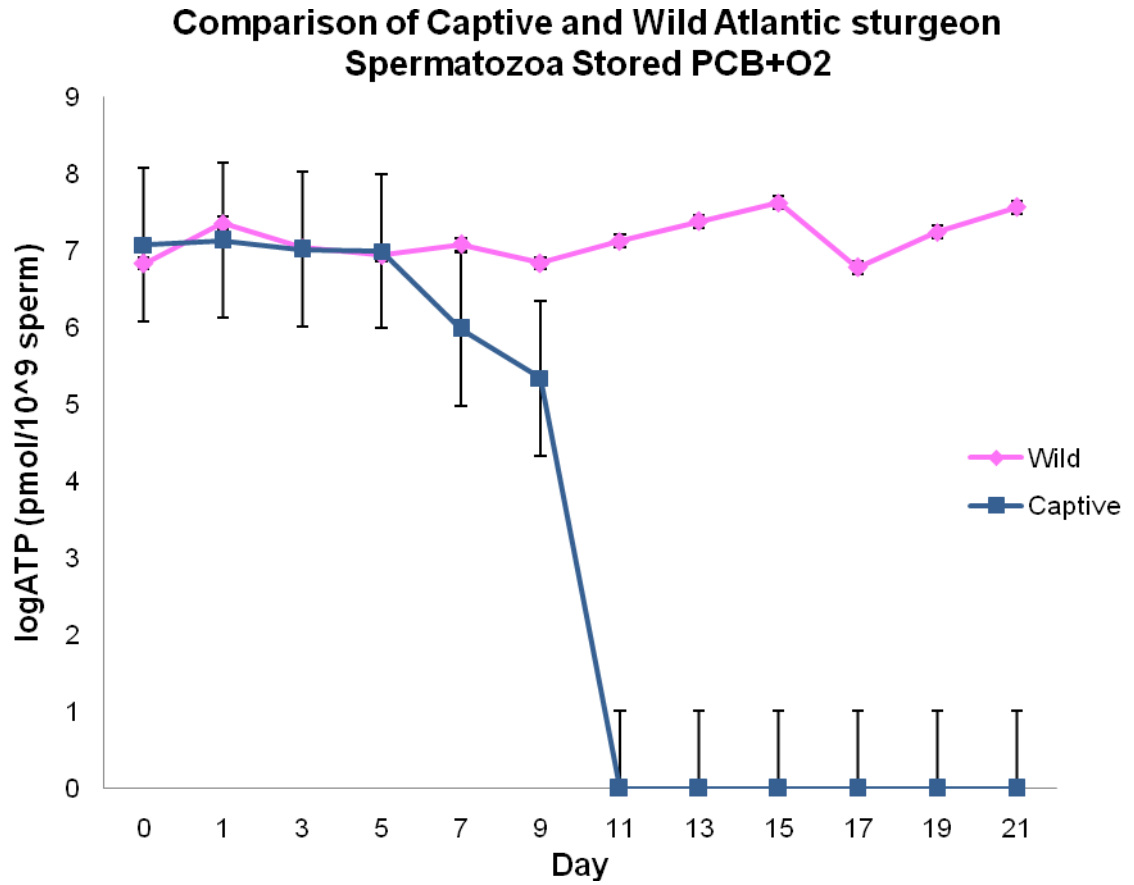


Figure 39. Differences in ATP levels (\pm SEM) between captive and wild Atlantic sturgeon spermatozoa stored diluted (PC extender) for 21 days with oxygen (2009).

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