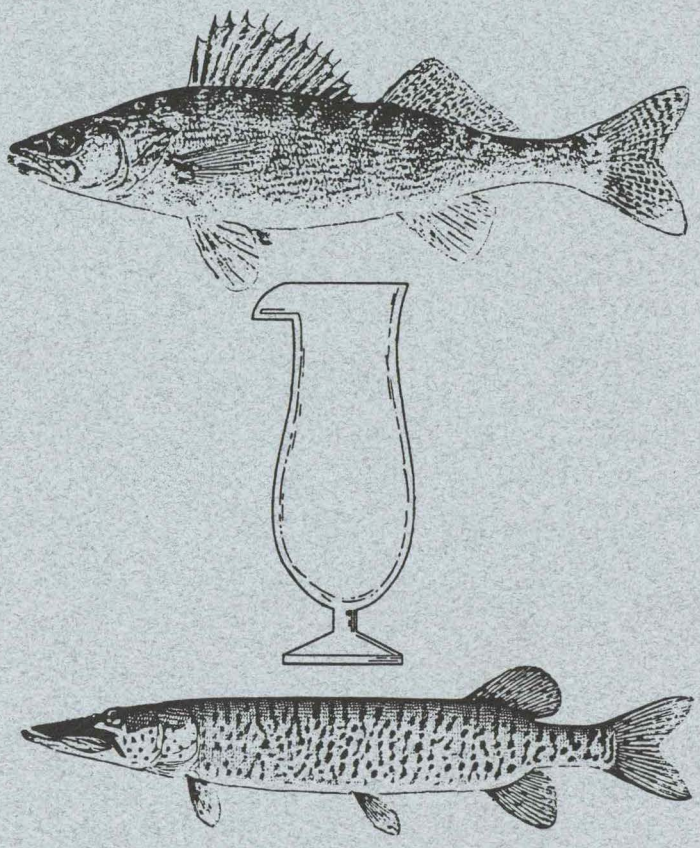
REFRIGERATED STORAGE AND CRYOPRESERVATION OF WALLEYE AND MUSKELLUNGE SEMEN

Technical Bulletin No. 4



Iowa Department of Natural Resources
Larry J. Wilson, Director

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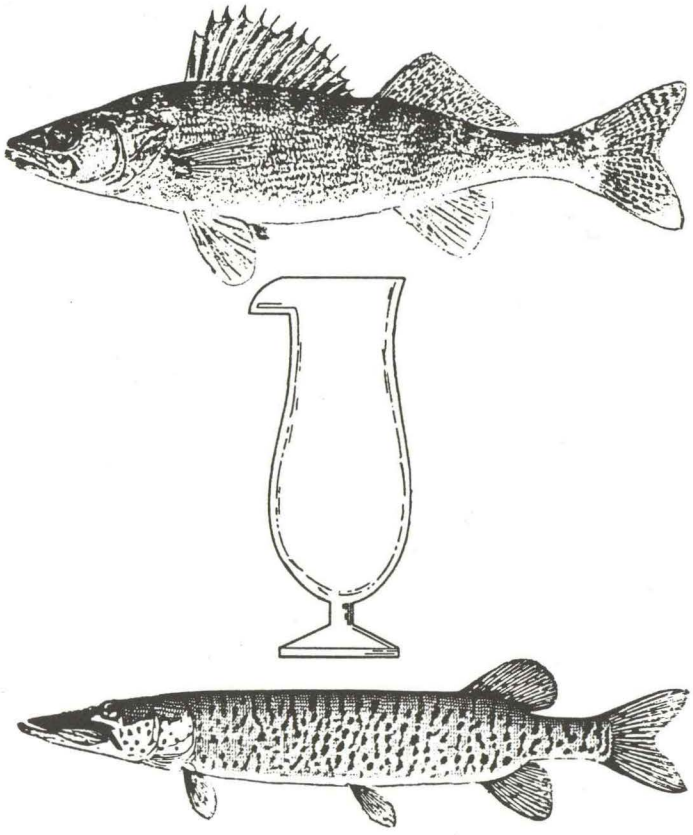

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ABSTRACT

The effects of additives, storage and temperature on cryopreservation of walleye *(Stizostedion vitreum vitreum)* and muskellunge *(Esox masquinongy)* semen were investigated. Chemical analyses of semen and information from the literature were used to characterize the chemical properties of muskellunge and walleye semen. This information was used as a guide to ionic composition of the extenders. Egg fertility was 90.8% with walleye semen mixed with an extender plus 180 μg/ml ampicillin and refrigerated 10-14 days, compared with 93.2% fertility of eggs fertilized with fresh semen. Significantly fewer viable eggs resulted from fertilization tests with frozen walleye semen when compared to fresh semen. Semen used after freezing, fertilized eggs at rates ranging from 34.4% to 83.2%, while use of fresh semen resulted in rates ranging from 83.2% to 98.4%. Egg viability associated with the use of refrigerated muskellunge semen was extremely variable but similar to control lots of eggs fertilized with fresh semen. Fertility ranged from 0 to 77% when eggs were fertilized with refrigerated semen, the fertility of control lots ranged from 1.3% to 76.0%. Frozen muskellunge semen fertilized ova at a maximum rate of 8.0%. Test results showed walleye and muskellunge semen can be stored by cryopreservation, but storage techniques and composition of the extender need to be improved.

Refrigerated Storage and Cryopreservation of Walleye and Muskellunge Semen

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INTRODUCTION

Cold storage of fish gametes has received intensive investigation the past 20 years. Much of this interest has been associated with salmonid fishes (Graybill 1968; Truscott et al. 1968; Graybill and Horton 1969; and Horton and Ott 1976) in the areas of semen extenders and cryoprotectants for storage of steelhead[1] *(Salmo gairdneri)* , Atlantic salmon *(Salmo salar),* and chinook salmon (*Oncorhynchus tshawytscha*) gametes. In 1980, Erdahl and Graham devised extenders and cryoprotectants for rainbow *(S. gairdneri)* and brown trout *(Salmo trutta)* semen. Earlier, Buyukhatipoglu and Holtz (1978) successfully stored undiluted rainbow trout semen for 15 days and obtained fertility rates as high as 81%. These investigators also found semen diluted 1:1 or 1:3 (semen to diluent) and frozen in pellets resulted in egg hatching rates of 6-80%. According to Legendre and Billard (1980), a semen dilution rate of 1:3 produced higher rates of fertility in rainbow trout eggs than did dilution rates of 1:1 or 1:9. Stoss and Holtz (1983c) successfully stored undiluted rainbow trout semen, supplemented with penicillin and streptomycin, with no loss of fertility. The semen was stored at 0°C in a moist oxygen atmosphere for 34 days and in polyvinylchloride bags for 20 days. They also demonstrated semen processing should occur immediately after it has been stripped from the males. If storage of unprocessed semen is unavoidable, semen should be kept in open vials and its depth should not exceed 5 mm (Stoss and Holtz, 1983a). Previous semen extenders have been mixtures of salts resembling fish seminal plasma, with or without the antifreezing protectants dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol. Cold storage temperatures have ranged from 5°C to -196°C. Fertility rates as high as 98% have been achieved (Erdahl and Graham 1980), but results have varied. The addition of Promine D (Central Soya) and bovine serum albumin to extenders, prior to freezing, produced significantly higher egg fertility rates when compared to extenders with no protein (Stoss and Holtz, 1983a). In other experiments, Stoss and Holtz (1983b) noted an increase in the DMSO concentration and a simultaneous increase in equilibration time resulted in a decreased rate of egg fertility.

Each spring, Iowa Department of Natural Resources fish hatchery personnel collect large numbers of male and female walleye *(Stizostedion vitreum vitreum)* and muskellunge *(Esox masquinongy)* for the purpose of propagation. Male fish become ripe several days before female fish and many are held in hatchery confinement for 2-3 weeks. This period of captivity results in thick, viscous semen and causes reduced fertility in male fish. Timely spawning of males and sperm preservation could enhance hatchery efficiency by reducing the number of males held, decreasing handling loss, increasing fertility, and reducing operational costs.

In 1982, investigations were started at the Rathbun and Spirit Lake Hatcheries to develop methods and techniques for preservation of walleye and muskellunge semen. The studies concentrated on techniques for both short-term (refrigerated) and long-term (frozen) storage of semen (Moore, 1983).

METHODS

General Semen Collection and Storage Techniques

Semen for this study was collected from male walleye and muskellunge using a water powered sink aspirator. Anesthetized fish were rinsed in fresh water and wiped dry. Semen was forced from the fish, held ventral side up, with gentle pressure along the gonads. Semen was aspirated into 10 ml vials and immediately cooled in an ice water bath. The composition of extenders used in semen storage were developed from the chemical properties of walleye sperm and seminal fluids described by Gregory (1970) and from chemical analysis of muskellunge sperm and seminal fluids performed at Iowa State University.

Short-term, refrigerated storage of sperm cells consisted of adding the extender to the semen at a rate of one part semen to two parts extender (volume to volume). No extender-semen equilibration time was allowed and a maximum storage depth of 2.0 mm per vial in-

[1](Current scientific name: *Salmo mykiss.*)

sured adequate oxygen exchange. Vials were stored unsealed in a refrigerator, the bottom portion of which contained water. Oxygen was bubbled through the water to provide a moist, oxygen rich environment and storage temperatures ranged from 1°C (33°F) to 5°C (41°F). Ampicillin, a broad spectrum antibiotic, was added to retard bacteria growth.

Long-term, frozen storage (cryopreservation) of sperm cells required a modification of the extender and the testing of dimethyl sulfoxide (DMSO), ethylene glycol (EG), mannitol, bovine serum albumin (BSA), PRO-FAM soy protein, sucrose, and egg yolk as freeze protectants. The modified extenders were mixed with semen using different ratios and equilibration times before freezing. The semen-extender mixtures were aspirated into 0.25 ml plastic straws, sealed, and placed on dry ice for 30 minutes or one ml was placed on dry ice to form pellets. Samples were stored in liquid nitrogen a minimum of 24 hours. Straws and pellets were thawed at temperatures ranging from 5°C (41°F) to 43°C (110°F) and sperm cells evaluated for viability and fertility. Sperm cell activity was evaluated using a motility scale of zero to 5; zero indicated no motility and 5 indicated vigorous movement ([1]Hoyle, Truscott, Idler 1967). Approximately 0.1 ml of semen was placed on a microscope slide, activated with water and examined under a compound microscope.

The ability of sperm cells to fertilize eggs was evaluated using equal volumes of eggs taken from two females. Eggs were taken by the "dry method" of spawning. Following the addition of semen to the egg mass, fertilization was initiated by adding water. Eggs and semen were then swirled gently for 3 minutes. Walleye eggs were stirred in a fuller's earth solution for 5 minutes to prevent clumping. Muskellunge eggs were rinsed several times in fresh water but not treated with clay solution. Eggs from both species were water hardened 4 hours, then incubated in standard flow-through hatching jars. To reduce variability in fertility due to semen quality, semen from at least two males was mixed and used in each trial. Replicates were performed for each experimental semen lot; and, control lots of eggs were fertilized with fresh semen.

To determine egg viability, 3 to 4 days after fertilization, aliquots of 125 eggs were sampled from each test jar and examined under a dissecting microscope. Statistical analyses on counts of live and dead eggs were completed by chi-square testing at the 0.05 probability level.

Walleye Semen Evaluation

In the preliminary evaluation of the viability of refrigerated walleye semen, small lots of eggs, ranging from 118 ml (4 oz) to 236 ml (8 oz), were fertilized with 0.67 ml semen mixed with 1.33 ml extender. Refrigeration time ranged from 1 day in 1982 to 18 days by 1985.

Egg fertility tests were expanded in 1985 and by 1986 included approximately one-half the eggs taken at Rathbun Hatchery and 5% of the eggs at the Spirit Lake Hatchery. Semen used during these evaluations was refrigerated 2 to 14 days. Normal hatchery egg taking procedures were followed and testing was conducted using a random and uniform egg selection format. Uniform testing consisted of two equal lots of eggs dry stripped from one female. One lot was fertilized with refrigerated semen and the second lot fertilized with fresh semen. The semen used to fertilize each lot of eggs was taken from two male walleye. During random testing, females were randomly selected, stripped, and eggs fertilized with either refrigerated or fresh semen. More than 3.0 ml of fresh semen, from the two male walleye, was used to fertilize each control egg lot, while 2.4 to 3.0 ml of refrigerated semen, mixed with extender, was used per experimental egg lot.

During cryopreservation of semen, DMSO and ethylene glycol were added to previously mentioned sugar and protein extender components in an effort to improve sperm cell wall integrity. DMSO was evaluated at 5%, 7%, and 10% of extender volume and ethylene glycol at 7%. A combination of 13 extenders and cryoprotectants were tested on egg lots ranging from 59 ml (2 oz) to 437 ml (16 oz). Semen volume was 0.67 ml, diluted in 1.33 ml extender and frozen from 1 to 13 days.

Muskellunge Semen Evaluation

The formulated muskellunge extender and walleye extender were both evaluated for mus-

[1]Hoyle, R. J., B. Truscott and D. R. Idler. 1967. Studies on Freezing Sperm of Atlantic Salmon *(Salmo salar)*. Fisheries Research Board of Canada, Technical Report No. 93.

kellunge semen refrigeration trials in 1982 and 1983. During the years following, the walleye extender proved more successful and six variations of the extender, adjusted to a pH of 8.8, were used for short-term preservation tests. Egg volumes ranged from 118 ml (4 oz) to 236 ml (8 oz) and total semen volume was 0.5 ml mixed with 1.0 ml extender.

Cryopreservation of muskellunge semen, in 1982 and 1983, concentrated on the fertilizing capabilities of sperm frozen 24 hours and thawed at 5°C (41°F) and 16°C (61°F). The studies were expanded to include motility of sperm cells and manipulation of thawing temperature from 1984 through 1987. DMSO and EG were evaluated at the 7% concentration level, and equilibration times ranged from 5 to 15 minutes at semen to extender dilution ratios of 1:3, 1:5, and 1:9. Egg lots ranged from 59 ml (2 oz) to 437 ml (16 oz). Control eggs were fertilized with 0.25 ml fresh semen and test egg lots with this amount diluted as above.

FINDINGS

From the muskellunge semen analysis and the walleye seminal properties reported by Gregory (1970), extenders were developed for the preservation of walleye and muskellunge semen (Table 1).

Walleye

In 1982 fresh semen produced slightly higher egg viability when compared to extended semen refrigerated 24 and 48 hours. Percent egg viability was 77.0 and 79.0 for semen stored 24 and 48 hours and 84.0 and 89.0 for fresh semen. In 1983, motility rating of refrigerated raw semen dropped to stage 2 (very few fast swimmers) after 4 days, while extended semen was at stage 4 (most swimming vigorously) after 9 days. Eggs fertilized with fresh semen had slightly higher viability rates than that of eggs fertilized with extended semen, refrigerated up to 9 days. The pooled means, however, were not significantly different ($P>0.05$) (Table 2). As the storage period lengthened, ampicillin aided in maintaining the integrity of the sperm cells and egg viability increased compared to that of eggs fertilized with semen preserved in extender only. In 1984, mean egg viability produced by fresh semen was 93.2% compared to 90.8% and 86.4% for semen extended and refrigerated 10 to 14 days and mixed with ampicillin at 180 and 3,325 µg/ml of volume, respectively (Table 3). Differences between fresh semen and extended semen, which contained the lower ampicillin concentration,

Table 1. *Composition of the extender added to walleye, and muskellunge semen used in cryopreservation trials at Rathbun and Spirit Lake Fish Hatcheries.*

Constituent	Walleye[a] Extender	Muskellunge[a] Extender
Calcium chloride dihydrate (CaCl2.2H2O)	0.234 g	0.302 g
Magnesium chloride (MgCl2.6H2O)	0.267 g	0.464 g
Sodium phosphate dibase (Na2HPO4)	0.472 g	0.471 g
Potassium chloride (KCl)	3.744 g	2.918 g
Sodium chloride (NaCl)	13.155 g	8.820 g
Glucose	20.000 g	20.000 g
Citric acid monohydrate (HOCCOOH [CH2COOH]2.H2O)	0.200 g	0.200 g
Sodium hydroxide (NaOH) solution[b]	40 ml	—
Potassium hydroxide (KOH) solution[c]	—	20 ml
Bicine solution[d]	40 ml	40 ml

[a]All ingredients are added to 1,920 ml distilled water.

[b]NaOH solution is 1.27 g NaOH/100 ml water.

[c]KOH solution is 1.27 g KOH/100 ml water.

[d]Bicine (N,N-bis[2-hydroxyethyl]glycine) solution is 5.3 g bicine/100 ml water.

Table 2. Percent viability of walleye eggs at 3 days post-fertilization at Rathbun Fish Hatchery, 1983.*

Days of Semen Storage	Trial	Refrigerated Semen Extender with Ampicillin Concentrations of 3,325 µg/ml	180 µg/ml	Extender Only	Fresh Semen
3[b]	1	90.4	----	90.4	80.8
	2	85.6	68.0	68.0	64.0
4	1	86.4	80.0[a]	73.6[a]	93.6
	2	84.8	80.8	89.6	88.0
5	1	93.6	91.2[a]	97.6	98.4
	2	89.6[a]	92.0[a]	89.6[a]	98.4
6	1	81.6[a]	86.4[a]	92.8[a]	98.4
	2	85.6[a]	89.6	93.6	95.2
7	1	68.8	60.8	57.6	64.0
	2	92.8	96.8	93.6	98.4
8	1	63.2[a]	78.4[a]	79.2[a]	96.8
	2	79.2[a]	84.0[a]	84.0[a]	96.8
8 (pH 8.4)		92.0	85.6	83.2	89.6
8 (pH 9.0)		88.8	82.4	75.2[a]	89.6
9	1	44.0[a]	80.0[a]	0.0[a]	97.6
	2	13.6[a]	20.0[a]	1.6[a]	48.0
9 (pH 8.4)		79.2	86.4	69.6[a]	83.2
9 (pH 9.0)		84.0	80.8	59.2[a]	83.2
Mean of Pooled Data		77.6	78.8	72.0	86.8

[a] Significantly less than fresh semen control (P <0.05).
[b] Extender pH is 7.83 unless noted differently.
*236 ml (8 oz) egg volume

Table 3. Percent viability of walleye eggs at 3 days post-fertilization at Rathbun Fish Hatchery, 1984.*

Days of Semen Storage	Trial	Refrigerated Semen in Extender with Ampicillin Concentrations of 3,325 µg/ml	180 µg/ml	Fresh Semen
10	1	94.4	92.8	97.6
	2	90.4[a]	94.4	96.8
11	1	93.6[a]	96.0	99.2
	2	83.2[b]	88.0	86.4
12	1	79.2[a,b]	88.0	88.0
	2	94.4	90.4	93.6
13	1	91.2[a]	96.0	98.4
	2	90.4	85.6	91.2
14	1	77.6[a,b]	95.2	96.8
	2	74.4	83.2	84.0
Mean of Pooled Data		86.4[a]	90.8	93.2

[a] Significantly less than fresh semen control (P <0.05).
[b] Significantly less than 180 µg/ml test group (P <0.05)
*118 ml (4 oz) egg volume

were not significant (P>0.05). Fresh semen, however, provided a significantly higher rate of egg viability (P<0.05) than the semen extender mixture containing 3,325 µg/ml ampicillin. When the two ampicillin rates were compared, egg viability was significantly higher (P<0.05) when the lower dosage was used.

Similar results were found in 1985 and 1986 during a test in which semen was extended and refrigerated 14 to 18 days (Table 4). A comparison of eggs fertilized with fresh semen (79.7%) and those fertilized with preserved semen plus 370 µg/ml ampicillin (68.9%) revealed no significant difference (P<0.05) in viability; however, semen mixed with extender plus 1,650 µg/ml ampicillin produced only 51.6% viable eggs, a significant reduction (P<0.05). Results also indicated extended refrigerated semen began to deteriorate after 16 days of storage.

Extended, refrigerated semen was used to fertilize 10 million eggs in two uniform hatchery production comparisons, 1985 to 1987 (Tables 5 and 6). Egg viability obtained with extended,

Table 4. Percent viability of walleye eggs at 3 days post-fertilization at Rathbun Fish Hatchery, 1985 and 1986.

Days of Semen Storage	Trial	Refrigerated Semen in Extender with Ampicillin Concentrations of 370 µg/ml	1,650 µg/ml	Fresh Semen
14	1	87.2[a]	80.0[a]	94.4
	2	48.0	----	58.4
	3	55.2	----	58.4
15	1	88.0	83.2[a]	94.4
	2	72.0	----	77.6
	3	79.2	----	77.6
16	1	69.6[a]	11.2[a]	92.0
	2	70.4	----	75.2
	3	61.6[a]	----	75.2
17	1	90.4[a]	84.0[a]	96.8
	2	72.8	----	77.6
	3	49.6[a]	----	77.6
18	1	96.0	0.0[a]	11.4
	2	73.6[a]	----	85.6
	3	19.2[a]	----	85.6
Mean of Pooled Data		68.9	51.6[a]	79.7

[a]Significantly less than fresh semen control (P <0.05).

refrigerated semen was higher than eggs fertilized with fresh semen in the majority of tests but significant differences were found in only five comparisons (P<0.05). Results in the random production tests were similar, with a mean viability of 77.8% for extended semen and 75.0% for fresh semen (Table 7).

During tests of frozen semen in 1982 and 1983, the addition of 10% DMSO to the walleye extender did not produce more fertilized eggs than 7% DMSO. In 1982, frozen semen fertilized significantly fewer eggs (3% to 10%) than fresh semen (84.6%), and frozen pellets which contained semen, extender and either DMSO or EG as cryoprotectants were completely unsuccessful in fertilizing eggs. In 1983, semen, extender and EG, used with a 15 minute equilibration period, fertilized eggs at a 19% rate, but only a 10% rate was achieved without equilibration. A period of equilibration had the opposite impact on viability if semen-extender solutions contained DMSO. More viable eggs were produced without equilibration (20%) than with equilibration (11%). Viability of eggs fertilized with fresh semen ranged from 66.8% to 86.4%.

From 1984 through 1987, viability of eggs fertilized with extended semen frozen and stored in

Table 5. Mean percent viability of walleye eggs at 3 days post-fertilization during uniform production scale trials at Rathbun Fish Hatchery, 1985 through 1987.

Days of Refrigerated Semen Storage	Number of Trials	Extended Semen with 180 µg Ampicillin	Fresh Semen
	1985		
2	4	71.6	77.6
3	2	77.2	63.6[a]
4	2	86.8	76.8
5	4	81.0	79.6
6	2	82.0	73.2
10	2	81.6	76.4
14	1	76.8	73.6
14 + 4	2	72.0	61.2
	1986		
3	3	42.2	36.9
5	2	68.7	50.7[a]
6	1	80.0	74.6
	1987		
3	1	85.6	86.4
4	1	80.0	80.8
6	1	82.4	84.0
12	1	80.8	81.6

[a]Significantly less than corresponding extended semen group (P <0.05).

Table 6. Percent viability of walleye eggs at 3 days post-fertilization during uniform production scale trials at Spirit Lake Fish Hatchery, 1986 and 1987.

Days of Refrigerated Semen Storage	Trial	Extended Semen with 180 µg Ampicillin	Fresh Semen
1	1	74.7	74.7
	2	58.7	61.4
2	1	44.0	57.4
	2	86.6	58.6[a]
	3	93.3	69.3[a]
	4	84.0	49.3[a]
3	1	76.0	64.0
4	1	61.4	62.7
Mean of Pooled Data		72.5	62.2

[a]Significantly less than corresponding extended semen group (P <0.05).

Table 7. Percent viability of walleye eggs at 3 days post-fertilization during random production scale trials at Rathbun Fish Hatchery, 1985.

Days of Refrigerated Semen Storage	Trial	Extended Semen with 180 µg Ampicillin	Fresh Semen
4	1	78.4	81.6
	2	88.0	79.2
10	1	84.8	84.4
	2	88.0	84.8
11	1	73.6[a]	84.0
12	1	76.8	81.6
	2	90.4	38.4[a]
13	1	55.2[a]	69.6
	2	65.6	71.2
	3	67.2	66.4
14	1	88.8	84.0
Mean of Pooled Data		77.8	75.0

[a]Significantly less than corresponding trial group (P <0.05).

liquid nitrogen was significantly less (P<0.05) than eggs fertilized with fresh semen (Table 8). Of the extender cryoprotectant combinations evaluated, the six listed in Table 8 fertilized eggs consistently above 50%, while the other four did not. Semen preserved in extender No. 3 and thawed at 21.1°C (70°F) produced the most consistent results.

Muskellunge

Preliminary testing in 1982 and 1983 revealed the walleye extender with 20 g glucose/2 liters extender and no ampicillin preserved refrigerated muskellunge semen better than the specifically formulated muskellunge extender. Mean egg viability produced was 52.2% after 1 day of refrigeration, and 35.2% and 20% after 2 and 3 days, respectively. Fertility of eggs fertilized by fresh semen was 64.5%. Replacement of glucose with mannitol and the addition of 180 µg/ml ampicillin maintained sperm cell motility at stage 3 for 11 days, while walleye extender with glucose and ampicillin gave no motility after 3 days. Results in Table 9 show the viability of eggs fertilized with semen preserved in extender No. 1 was comparable to fresh semen in two tests and significantly greater (P<0.05) in a third. Semen stored in extenders Nos. 5 and 6 gave fair viability after 3 days of storage but were significantly less (P<0.05) than results obtained for fresh semen. Semen preserved in extender No. 4 fertilized fewer eggs than fresh semen after 3 days of storage; however, the number of eggs fertilized improved at days 4 through 6, and results were significant (P<0.05) at day 5.

In 1982, 8% of the muskellunge eggs were viable when fertilized with semen frozen in walleye extender and 7% DMSO, while fresh semen produced an egg fertility rate of 54.6%. In 1983, counts of eggs fertilized with frozen semen produced one fertile egg of 500 examined. The use of DMSO or EG with the walleye extender, with or without equilibration did not affect the outcome at thawing temperatues of 5°C (41°F) and 16°C (61°F). Mean

Table 8. *Percent viability of walleye eggs at 3 days post- fertlization during cryopreservation trials with semen frozen in different extender mixtures at Rathbun Hatchery, 1984 through 1987.*

Preservation Medium[a]	Trial	Thawing Temperature (°C)	% of Eggs Fertilized: Preserved Semen	% of Eggs Fertilized: Fresh Semen[b]
1	1	32.1	59.2	96.0
1	2	32.1	55.2	94.4
1	3	32.1	62.4	94.4
1	4	32.1	65.6	96.6
1	5	32.1	39.2	83.2
2	1	21.1	50.4	91.2
2	2	21.1	83.2	94.4
2	3	21.1	61.6	94.4
2	4	21.1	46.4	96.6
2	5	21.1	62.4	83.2
3	1	21.1	48.0	84.0
3	2	21.1	71.2	94.4
3	3	21.1	72.8	94.4
3	4	21.1	66.4	96.6
3	5	21.1	68.0	83.2
3	6	21.1	57.6	98.4
3	7	21.1	62.4	97.6
3	8	21.1	67.2	97.6
4	1	21.1	72.8	92.0
4	2	26.6	68.8	92.0
5	1	32.1	66.4	96.6
5	2	32.1	48.0	83.2
6	1	21.1	51.2	96.6
6	2	21.1	68.8	83.2
6	3	21.1	64.4	98.4
6	4	21.1	52.8	97.6
6	5	21.1	42.4	97.6

[a]Preservation media: 1) walleye extender with 15 g glucose/2 liters extender, 5 g mannitol/2 liters extender, 5% DMSO; 2) walleye extender with 15 g glucose/2 liters extender, 5 g mannitol/2 liters extender, 4 mg/ml BSA, 7.5 mg/ml PROFAM, and 5% DMSO; 3) same as No. 2 but with 7% DMSO; 4) walleye extender with 15 g glucose/2 liters extender, 5 g mannitol/2 liters extender, 4 mg/ml BSA, 7.5 mg/ml egg yolk 7% DMSO; 5) walleye extender with 15 g glucose/2 liters extender, 5 g sucrose/2 liters extender, 5% DMSO; 6) walleye extender with 15 g glucose/2 liters extender, 5 g sucrose/2 liters extender, 4 mg/ml BSA, 1.5 ml egg yolk/100 ml extender, and 7% DMSO.

[b]Significantly greater than frozen semen in all comparisons (P <0.05).

percent fertility of eggs fertilized with fresh semen was 41.

In 1984 eight extenders were mixed and muskellunge semen, frozen and evaluated for their ability to preserve motility. Sperm cells were thawed at six different temperatures. Results indicated rate of thaw impacted sperm viability and extender No. 2 should be further studied at a thaw rate of 26.2°C, No. 4 at 15.5 °C, No. 7 at 15.5°C, or 26.6 and No. 8 at 21.1°C or 26.6°C. Muskellunge semen that had been subjected to the rigors of freezing and thawing fertilized significantly fewer eggs ($P<0.05$) in 1985 and 1986 than did fresh semen and this was consistent in all but four tests. Fertility of eggs mixed with fresh semen ranged from 0.0% to 34.6%, while percent viability of eggs fertilized with frozen semen was 0.0% to 2.6% (Table 10).

In 1986, four new preservation media were formulated and evaluated for ability to maintain viability of sperm cells through stresses of freezing and thawing. Findings indicated extender No. 2a produced the highest number of motile sperm cells if thawed at 32.2°C (90°F), No. 7a at 21.1°C (70°F), No. 8a at 37.7°C (100°F), and No. 4a should be deleted. In 1987 fresh semen did a significantly better job fertilizing eggs than did frozen semen preserved in these extenders (Table 11). Only semen frozen in extender No. 8a was comparable to fresh semen.

DISCUSSION

Walleye semen, mixed with an extender plus antibiotics, re-

Table 9. *Percent viability of muskellunge eggs at 4 days post-fertilization during trials with refrigerated semen mixed with extender and ampicillin and stored one to six days at Spirit Lake Hatchery, 1984 and 1986.*

Preservation Medium[a]	Days of Semen Storage	Egg Volume (ml)	% of Eggs Fertilized: Preserved Semen	% of Eggs Fertilized: Fresh Semen
1	1	236	64.0	60.0
	1	236	77.2	65.2
	1	236	34.4[b]	18.4
4	3	118	13.3	76.0[c]
	4	118	4.0	1.3
	5	118	21.3[b]	5.3
	6	118	72.0	76.0
5	3	118	18.6	76.0[c]
	4	118	1.3	1.3
	5	118	0.0	5.3[c]
	6	118	0.0	76.0[c]
6	3	118	29.3	76.0[c]
	4	118	4.0	1.3
	5	118	1.3	5.3
	6	118	1.3	76.0[c]

[a]Preservation Media: 1) Walleye extender 10 g glucose/2 liters extender, 10 g mannitol/2 liters, 180 µg ampicillin; 4) Walleye extender with 5 g glucose/2 liters extender, 5 g mannitol/2 liters extender, 180 µg ampicillin; 5) Walleye extender with 7.5 g glucose/2 liters, 1.5 g mannitol/2 liters extender, 180 µg ampicillin; 6) Walleye extender with 5 g glucose/2 liters extender, 180 µg ampicillin.

[b]Significantly greater than control ($P<0.05$).

[c]Significantly greater than refrigerated semen ($P<0.05$).

Table 10. *Percent viability of muskellunge eggs at 4 days post-fertilization during cryopreservation trials with semen frozen in four extender mixtures at Spirit Lake Hatcherty in 1985 and 1986.*

Preservation Medium[a]	Trial	Thawing Temperature (°C)	% of Eggs Fertilized: Preserved Semen	% of Eggs Fertilized: Fresh Semen
2	1	26.6	0.0	0.0
	2	26.6	0.0[b]	34.6
	3	26.6	0.0[b]	34.6
4	1	15.5	1.3	0.0
	2	15.5	0.0[b]	10.6
	3	15.5	1.3[b]	34.6
	4	15.5	2.6[b]	34.6
7	1	15.5	0.6[b]	5.3
	2	26.6	1.3	5.3
	3	26.6	0.0[b]	34.6
	4	26.6	2.6[b]	34.6
8	1	21.1	0.6[b]	5.3
	2	26.6	2.0	5.3
	3	26.6	0.0[b]	34.6
	4	26.6	0.0[b]	34.6

[a]Preservation Media: 2) Walleye extender with 5 g mannitol/liter extender, 5 g glucose/liter extender, 4 mg/ml BSA, 7.5 mg/ml PRO-FAM, 5% DMSO - 1:9 dilution, 5 minute equilibration; 4) Walleye extender with 10 g mannitol/liter extender, 5 g glucose/liter extender, 8 mg/ml BSA, 7.5 mg/ml PRO-FAM, 7% DMSO - 1:3 dilution; 7) Same as No. 4 but a 1:9 dilution; 8) Same as No. 7 but with a 5 minute equilibration.

[b]Significantly less than fresh semen control ($P<0.05$).

Table 11. *Percent viability of muskellunge eggs at 4 days post-fertilization during cryopreservation trials with semen frozen in three extender mixtures at Spirit Lake Hatchery, 1987.*

Preservation Medium[a]	Trial	Thawing Temperature (°C)	% of Egg Fertilized: Preserved Semen	% of Egg Fertilized: Fresh Semen
2a	1	32.1	0.0[b]	12.0
	2	32.1	0.0[b]	12.0
	3	32.1	0.0[b]	8.0
7a	1	21.1	0.0[b]	12.0
	2	21.1	0.0[b]	12.0
	3	21.1	0.0[b]	8.0
8a	1	37.7	0.0[b]	12.0
	2	37.7	8.0	12.0
	3	37.7	4.0	8.0

[a]Preservation media: 2a) Walleye extender with 5 g glucose/liter extender,5 g sucrose/liter extender, 4 mg/ml BSA, 7.5 mg/ml PRO-FAM, 5% DMSO, 1:9 dilution, 5 minute equilibration; 7a) Walleye extender, 5 g glucose/liter extender, 10 g glucose/liter extender, 8 mg/ml BSA, 7.5 mg/ml PRO-FAM, 7% DMSO, 1:9 dilution; 8a) Same as No. 7a but with a 5 minute equilibration.

[b]Significantly less than fresh semen control ($P < 0.05$).

frigerated and oxygenated for 14 days, will fertilize eggs at rates similar to fresh semen. These results are consistent with findings of Stoss, et al. (1978) and Stoss and Holtz (1983c), who were able to maintain full fertility of rainbow trout semen for 34 days when stored in an oxygen rich environment and at cold temperatures. The lower concentration of ampicillin appears to be sufficient to prevent bacterial growth, while an increase in ampicillin concentration from 180 to 3,325 µg/ml reduced the ability to fertilize eggs. The relatively small decrease in fertility suggested a wide tolerance of egg and sperm to the antibiotic. Stoss and Refstie (1983) reported similar findings using penicillin and streptomycin.

When semen was stored beyond 14 days, egg viability declined and results became more variable. This variability may have been due to handling and storage techniques or extender properties. Adequate fertility can be achieved, however, to warrant use of this semen, if necessary. Uniform and random evaluation of walleye semen in a production mode revealed that preserved semen can fertilize eggs at a higher rate than that achieved with fresh semen. This is most likely a result of better semen distribution through the egg mass and a delay in semen activation caused by the extender.

Frozen walleye semen results were comparable to those reported by Horton and Ott (1976), and Buyukhatipoglu and Holtz (1978), and other researchers. The viability rate of walleye eggs using frozen sperm cells must be improved approximately 20% prior to use in hatchery production. Fertility of semen is high enough, however, to permit use of frozen sperm for crossbreeding of strains, transport of semen, long-term storage for emergency situations, and preservation of genetic materials.

Viability of muskellunge semen is very acceptable after 24 hours of refrigerated storage. Results were extremely variable after longer storage periods; however, excellent egg fertility was still achieved. Current use of semen preserved for 24 hours and less allows hatchery managers to "pre-strip" semen before eggs are spawned. This reduces the number of fish handled during spawning and the potential for contamination during fertilization. It also allows storage of unused semen for use the following day. Preservation medium No. 4 should be given additional consideration for long-term storage applications.

Frozen muskellunge semen produced unacceptably low egg viability rates (2%), which is not consistent with those found by Koldras and Moczarski (1983) for northern pike. These investigators achieved rates of 35% to 94% at 2 days but achieved no viable fry at hatch. The investigators cite low water temperatures was the reason.

A final observation of cryopreserved sperm cells is that motility is not necessarily an indicator of fertility. Results in this investigation showed sperm motility rates of 3 to 4, yet the actual fertilizing ability of these cells was poor to nonexistent. Egg viability was 2.6% or less with cryopreserved semen, but was 5.3 to 34.6% with fresh semen. Continued experimentation with freezing and thawing techniques is extremely important to accomplish desired levels of egg fertilization.

RECOMMENDATIONS

1. The composition of the walleye semen extender listed in Table 1 should be used as a basis to preserve walleye and muskellunge semen for short periods.
2. The use of walleye semen mixed with extender containing 180 μ ampicillin/ ml of semen-extender mixture and refrigerated from one to 16 days should become a routine part of the walleye egg taking program of department hatcheries.
3. Cryopreservation of walleye semen should be consigned to emergency semen storage, preservation of genetic material over an extended period, and transportation of semen for the crossbreeding of strains.
4. The use of muskellunge semen mixed with extender No. 1 or extender No. 4 and refrigerated from 1 hour to 2 days, and up to 6 days, respectively, should become a part of the muskellunge egg taking program of department hatcheries.
5. The use of frozen muskellunge semen is not recommended at this time.
6. Continue experimentation and development of extenders used to refrigerate and freeze muskellunge and walleye semen.

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