A suggested protocol for extending milt from male salmonids for use in wild spawn operations

October 31, 2010

Kevin B. Rogers, Aquatic Research, Colorado Division of Wildlife, PO Box 775777, Steamboat Springs, CO 80477, kevin.rogers@state.co.us

---

Overview

Recent interest in creating sterile hybrids (e.g. tiger trout or splake) for the Colorado Division of Wildlife has created a need to easily transport viable gametes from one body of water to another as parents often come from distant locations. The simple process of extending milt allows culturists the convenience of not having to transport live adult fish. Extended milt can also be used to easily enhance genetic diversity in bottlenecked native cutthroat trout brood stocks or help achieve adequate fertilization rates when males are scarce. The latter condition is surprisingly common in wild spawn operations, as male salmonids often arrive on the spawning grounds before females, and are often unable to produce much milt during the tail end of the run. Being able to dilute what little milt they do produce to allow fertilization of large lots of eggs can be a useful asset for these operations.

Extending trout milt

Collecting milt

Trout semen activated by dilution with water has an extremely short life spawn with almost 90% of the motility subsiding after just 10 seconds of exposure. As such, it is extremely important that no water find its way into the extending process. Drying the vent area of ripe males with blue paper shop towels provide an inexpensive way to rapidly soak up any excess water. Males can then be stripped into a small glass to collect the semen, making sure that it is not contaminated with feces or urine. If males are not limiting, contaminated samples should be discarded immediately and a new extraction initiated.

FIGURE 1: Milt stripped from a male brook trout after drying the vent area.

Extending milt

We have had good success using a ratio of 1 part trout semen to 6 parts extender developed to preserve walleye semen (Moore 1987, Satterfield 1992). Although others have suggested that less complex formulae work best (Scott and Baynes 1980), preliminary research suggests that better motility and longer storage times can be achieved with the recipe in Table 1. If ripe males are not limiting, higher concentrations of milt can be used without penalty. Of perhaps greater concern is the need to keep the milt well oxygenated. Storage vessels must therefore allow as much surface area to be exposed as possible. Fifty mL tissue culture flasks (e.g. VWR# 82051-076) are recommended, and can be preloaded with 3 mL of extender prior to heading out in the field so that they can be properly chilled. An adjustable pipettor can then be used to pull 0.5 mL milt out of the small glass and into the extender. When laid flat, this volume will allow the layer of extended milt to be no more than 2 mm thick which will help ensure adequate oxygenation needed for preservation.

TABLE 1: Extender composition (from Moore 1987)

Constituent	Quantity[a]
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0.234g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.267g
Sodium phosphate dibase (Na_2HPO_4)	0.472g
Potassium chloride (KCl)	3.744g
Sodium chloride (NaCl)	13.155g
Glucose	20.000g
Citric acid monohydrate ($HOCCOOH[CH_2COOH]_2 \cdot H_2O$	0.200g
Sodium hydroxide (NaOH) solution[b]	40 mL
Bicine solution[c]	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]Bicine (N, N-bis[2-hydroxyethyl]glycine) solution is 5.3 g bicine/100 mL water.

Sample storage

Oxygenation

Oxygen is critical to maintain viability of the milt, so the air above the milt layer in the culture flasks should be replaced with medical grade oxygen readily found from hospital suppliers. Regulators can be fitted with tubing and air nozzles available at most hardware stores that carry supplies for compressed air tools. Make sure to vent any excess pressure built up in the nozzle before inserting it into the vial of extended milt. The oxygen atmosphere over the milt should be replaced daily to maximize viable storage time.

FIGURE 2: Oxygenating extended milt in tissue culture flask

Temperature

Semen viability is a function of temperature (Jensen and Alderdice 1984), so milt should be kept as cool as possible without allowing it to freeze. Laid flat in the bottom of a cooler, between layers of newspaper or bubble wrap can ensure the extended milt is as close to 0ºC without freezing as possible. Ice packs are preferred over crushed ice as the latter ultimately provide another source of water that could potentially activate the sperm.

Viability

The methods described above can maintain viable walleye semen for up to ten days (Satterfield 1992), but motility in salmonid sperm does not seem to last quite as long. If storage for more than four days is required, alternative extender recipes should be considered. Stoss and Holtz (1983) were was able to keep rainbow trout milt viable for up to 34 days when antibiotics were added, with acceptable fertilizing capacity for up to 20 days. McNiven et al. (1993) found that using a non-aqueous fluorocarbon emulsion also greatly enhanced viable storage times as well. Exended milt can be stored indefinitely with cryopreservation (Horton and Ott 1976, Stoss and Refstie 1983, Glogowski et al. 2000).

Use

Recommendations on dose vary widely among authors, but with one mL of milt containing 20 billion sperm, it is clear that not much is needed to achieve adequate fertilization rates (Scott and Baynes 1980). We had good results making tiger trout at North Delaney Butte Lake with 1 mL of extended milt for 2500 eggs. That would suggest that each tissue culture flask should be able to fertilize close to 10,000 eggs. Ideally, eggs should be fertilized in the ovarian fluids prior to dilution with water.

Acknowledgments

Steve Brinkman is thanked for preparing several extender solutions evaluated during the development of this protocol. Useful input and manuscripts were provided by Steve Brinkman, Kay Knudsen, George Schisler, Keith Koupal, and Ken Kehmeier.

Literature cited

Glogowski, J, M. Kwasnik, B Piros, K. Dabrowski, K. Goryczko, S. Dobosz, H. Kuzminski, and A. Ciereszko. 2000. Characterization of rainbow trout milt collected with a catheter: semen parameters and cryopreservation success. Aquaculture Research 31:289-296.

Horton, H. F., and A. G. Ott. 1976. Cryopreservation of fish spermatozoa and ova. Journal of the Fisheries Research Board of Canada 33:995-1000.

Jensen, J. O. T., and D. F. Alderdice. 1984. Effect of temperature on short-term storage of eggs and sperm of chum salmon (*Oncorhynchus keta*). Aquaculture 37:251-265.

McNiven, M. A., R. K. Gallant, and G. F. Richardson. 1993. Fresh storage of rainbow trout (*Oncorhynchus mykiss*) semen using a non-aqueous medium. Aquaculture 109:71-82.

Moore, A. A. 1987. Short-term storage and cryopreservation of walleye semen. The Progressive Fish-Culturist 49:40-43.

Satterfield, J. R. 1992. Walleye semen collection, preservation, and storage. Doctoral dissertation, Colorado State University, Fort Collins.

Scott, A. P., and S. M. Baynes. 1980. A review of the biology, handling and storage of salmonid spermatozoa. Journal of Fish Biology 17:707-739.

Stoss, J., and W. Holtz. 1983. Successful storage of chilled rainbow trout (*Salmo gairdneri*) spermatozoa for up to 34 days. Aquaculture 31:269-274.

Stoss, J., and T. Refstie. 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. Aquaculture 30:229-236.