Recent advances in cryopreservation of salmonid fish semen

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Justification for the studies

Poor performance of published protocols, low post-thaw quality of semen and a very short recommended time for fertilization (30 s).

Promising preliminary results indicating good post-thaw rainbow trout sperm quality with the use of glucose-methanol extender.
Justification for the study
Low quality of cryopreserved semen.

Figure 3. Fertilization (mean ± SEM) of eggs using fresh (closed squares) or cryopreserved (open squares) Pacific oyster sperm. Three pools of sperm were collected, cryopreserved and assayed independently. The same batches of eggs were used with the fresh and thawed sperm. (Modified from Adams et al. 2004).

Cryopreservation of rainbow trout semen using four different extenders

I 0.3 M glucose, 10% methanol; II 0.3 M glucose, 10% DMSO

Methanol – permeating cryoprotectant
Glucose – nonpermeating cryoprotectant
Glucose concentration in the extender is important for the cryopreservation of rainbow trout semen.

Dilution: 1:3
Effects of 1:3 and 1:5 sperm-to-extender dilution ratios on sperm motility parameters of fresh and cryopreserved semen

- Motility (%)
- VCL (μm/s)
- VAP (μm/s)
- VSL (μm/s)
- LIN (%)
- ALH (μm)

*Significant differences between groups.
Sperm motility characteristics and fertilization rates of fresh and cryopreserved semen

Post-thaw motility – 49.9 ± 6.8%

Mean sperm concentration and osmolality of fresh undiluted semen were 10.87 ± 2.48 x 10^9 spermatozoa and 251 ± 39 mOsm/kg, respectively.
Brown trout (*Salmo trutta* m. *fario* L.)

- a major source of freshwater fish resources in Europe because of its commercial value for aquaculture and extreme importance for angling.
- naturally subdivided into a large number of reproductively isolated and genetically distinct populations
The **tiger trout** (*Salmo trutta X Salvelinus fontinalis*) is a sterile, **intergeneric hybrid** of the **brown trout** (*Salmo trutta*) and the **brook trout** (*Salvelinus fontinalis*).
Brook trout (*Salvelinus fontinalis*; Mitchell)

Important commercially, recreationally, and ecologically in Europe. It is of interest in aquaculture because it is almost completely resistant to the viral hemorrhagic septicemia virus and can easily be subjected to genome manipulation.
Sparctic Char: Strange Nighttime Saltwater Spawners from Europe!

December 3, 2011 By JD — 4 Comments

Spawning pair of Sparctic Char in Holland's Lake Oostvoorne (Janny Bosman photo)
Materials and Methods

Source of milt
Brown trout (n=9); 3 years of age
Brook trout (n=9; 2 years of age

Cryopreservation
0.2M glucose in 9% MeOH;
Dilution 1:5 in 0.25 ml straws;
15 min equilibration,
Thawing 40ºC, 5 s.

Fertilization
Brown trout 1; 3; 6×10⁵ sperm/egg ratio
Brook trout 3; 6×10⁵ sperm/egg ratio
Fertilization rates were measured at the eyed and hatched stages.

Measurements of sperm motility and concentration
Sperm motility - measured in fresh semen after dilution and in frozen samples.
Sperm concentration - measured using Nucleocounter SP-100.
Sperm collection using a catheter
Collection of whitefish
European huchen
Reproductive system of sex-reversed females of rainbow trout
CASA analysis of sperm motility
Extension of semen with glucose-methanol extender
Equilibration of straws filled with extended semen
Equilibration of straws filled with extended semen
Freezing
Thawing
40 °C, 5 s
Effect of cryopreservation on sperm motility parameters

Brown trout  

Brook trout
Effect of sperm-to-egg ratio on fertility of post-thaw cryopreserved sperm

Brown trout

Brook trout

Fertilization rate (%)
Effect of post-thaw sperm storage on motility parameters

Brown trout

![Diagram showing motility, VCL, VSL, VAP, LIN, and ALH parameters for Brown trout before and after 60 minutes of storage.]

Brook trout

![Diagram showing motility, VCL, VSL, VAP, LIN, and ALH parameters for Brook trout before and after 60 minutes of storage.]

- **Motility (%)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.

- **VCL (μm s⁻¹)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.

- **VSL (μm s⁻¹)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.

- **VAP (μm s⁻¹)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.

- **LIN (%)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.

- **ALH (μm)**
  - 0 min vs 60 min for Brown trout and Brook trout.
  - Bars compared with letters 'a' and 'b' indicating statistical significance.
Effect of post-thaw sperm storage on motility parameters

It had been assumed that thawed semen must be used immediately for fertilization within 30-second sperm storage after thawing significantly reduces the fertilization rate.

\[ \frac{30}{5s} = 6 \text{ straws} \]

\[ 60 \text{ (min)} \times 60 \text{ s} = 3600 \text{ s} / 5s = 720 \text{ straws} \]

Prolonged handling time of brook trout thawed semen could lead to better organization of hatchery work because the thawing procedure of several sperm samples for fertilization trials is time consuming.
Technical Note
The use of concentrated extenders to improve the efficacy of cryopreservation in whitefish spermatozoa
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Cryopreservation of rainbow trout semen using a glucose-methanol extender
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Efficient method for cryopreservation of European huchen (Hucho hucho L.) and grayling (Thymallus thymallus L.) semen
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Application of glucose–methanol extender to cryopreservation of semen of sex-reversed females rainbow trout results in high post-thaw sperm motility and fertilizing ability
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Effect of postthaw storage time and sperm-to-egg ratio on fertility of cryopreserved brook trout sperm
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Implementation
Further experiments

Different species

Improvement of technology

• Addition of antioxidants (cysteamine, glutathione, etc., antioxidative enzymes)
• Different sugars (sucrose, trehalose, etc.)
• Anti-freeze proteins
• Potassium ions
• Buffers
• Higher volume of straws
• Higher sperm concentrations in straws
A - whitefish

- Motility (%)
  - Fresh: 80
  - Equilibrated: 80
  - Cryopreserved: 60

- VCL (μm/s)
  - Fresh: 150
  - Equilibrated: 150
  - Cryopreserved: 150

- VAP (μm/s)
  - Fresh: 50
  - Equilibrated: 100
  - Cryopreserved: 100

B - Northern pike

- Motility (%)
  - Fresh: 80
  - Equilibrated: 80
  - Cryopreserved: 80

- VCL (μm/s)
  - Fresh: 150
  - Equilibrated: 150
  - Cryopreserved: 150

- VAP (μm/s)
  - Fresh: 120
  - Equilibrated: 120
  - Cryopreserved: 120
Preliminary results
16 October 2015

Cryopreservation of brown trout, cherry salmon and white-spotted char semen

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Joanna Nynca
Mariola Dietrich
Konrad Ocalewicz

Nanae Fresh-Water Station
Director Etsuro Yamaha
Brown trout

![Graph showing sperm motility for different preservation methods: Fresh, Equilibrated, Thawed, with corresponding mean and SD values.]

<table>
<thead>
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<th></th>
<th>Fresh</th>
<th>Equilibrated</th>
<th>Cryopreserved</th>
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**Mean**
- Fresh: 65.4%
- Equilibrated: 55.4%
- Cryopreserved: 52%

**SD**
- Fresh: 10.74
- Equilibrated: 13.24
- Cryopreserved: 16.05
Cherry salmon *Oncorhynchus masou*
Very thick semen, observed agglutination of spermatozoa after addition of extender to the fresh semen and in the thawed semen. Fish were at the end of spawning season.
White spotted char *Salvelinus leucomaenii*
White spotted char

<table>
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<th>Male No.</th>
<th>Fresh</th>
<th>Equilibrated</th>
<th>Cryopreserved</th>
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<tr>
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Agglutination of spermatozoa after addition of extender and after thawing. Motility of the thawed sperm was short - 2-3 sec. *Fish were sampled one day before experiment.*
Effect of supplementation of glucose-methanol extender with potassium ions on sperm motility of salmonid fish
Replacement of glucose with trehalose or sucrose in GM extender

Fig. 1. Cryopreservation of A – salmon, B – brown trout, C – brook trout, D – sex-reversed female rainbow trout, E – whitefish semen with 9% methanol extender containing 0.18M glucose; 0.18M sucrose; and 0.18M trehalose.
Summary

GM extender seems to be well suited for cryopreservation of salmonid fish semen.

Species specific modification may be necessary. For example trehalose for whitefish.

The possibility of post-thaw semen storage for the prolonged time (at least 60 min) as well as the obtainment of high fertilization rate at low sperm-to-egg ratio can lead to the significant improvement in implementation of cryopreservation in hatchery practice.

Further studies should be focused on scaling up this efficient cryopreservation technique for application in hatchery conditions.

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