# Preservation of *Cirrhinus microlepis* sperm

Sombut SINGSEE[*], Unnop IMSILP, Ponlachart PEWNANE and Naruepon SUKUMASAVIN

Aquaculture of Indigenous Mekong Fish Species Component, MRC Fisheries Programme

## ABSTRACT

Preservation of fish milt begins with collecting milt by either striping or dissecting for testis. The milt is preserved in a short – storage (4-9 °C) by either keeping fresh milt or diluted milt with variety of solutions. In the short – storage, milt which was diluted 1:5 with Modified Cortland solution can be kept for 48 hrs. Efficiency of the milt after preservations as determined by sperm motility, fertilization rate, hatching rate and survival rate were 30, 70.88±5.03, 75.59±4.50 and 77.87±7.92 %, respectively. For long-storage, or cryopreservation at -196°C, milt was diluted 1:5 with 10 % milk + 2.5 % glucose + 10 % methanol equilibration period at 4 ċ for 15 minutes and then diluted milt to freezing rate at -2.95 °C/min or cane#1 for 20 minutes before plunge into liquid nitrogen. Efficiency of the milt after preservations at 2 hrs as determined by sperm motility, fertilization rate, hatching rate and survival rate were 30, 62.66±17.59, 67.04±25.85 and 63.66±20.10 %, respectively. The efficiency of the preserved milt decreases in the longer preservation period, different species, methods and diluted solutions. If effective cryopreservation can be developed as good as in livestock cryopreservation, then the fish propragation of Thailand would be advanced in the future.

KEYWORDS : preservation, milt, *Cirrhinus microlepis*

## INTRODUCTION

Techniques of sperm management have been established in several freshwater fish species such as cyprinids (Billard *et al*., 1995), siluroids (Legendre *et al*., 1996) and in salmonids (Scott and Baynes, 1980; Billard, 1992). Among these techniques, sperm storage and cryopreservation are of special interest. At 0°C conditions, spermatozoa can be stored for a few hours up to several days, depending on the species while cryopreserved gametes can be theoretically stored between 200 and 32,000 years without deleterious effect (Ashwood-Smith, 1980).

The use of cryopreserved spermatozoa can be delayed from the date of collection and adjusted to the moment of ova processing. The benefits of this technique include:

- Synchronization of gamete availability of both sexes: ovulations are only noticed when sperm production declines in cross fertilization of different strains like spring and autumn spawning herring, *Clupea harengus* L. (Blaxter, 1953).
- Use of the total volume of available semen: this is useful for sperm economy in species where semen is difficult to obtain i.e., Japanese eel, *Anguilla japonica* Temminck and Schlegel) (Ohta and Izawa, 1996), but also in species where only low volume of semen can be stripped in captivity i.e., yellowtail flounder, *Pleuronectes ferrugineus* L. (Clearwater and Crim, 1995) or turbot, *Psetta maxima* L. (Suquet et. al., 1994).
- Simplifying broodstock maintenance: off season spawning can be induced in most cultured fish species by the manipulation of photoperiod and temperature cycles

* if_napanom@yahoo.com

(Bromage, 1995). However, the technique is cost intensive. When cryopreserved sperm is available all year round, the manipulation of the spawning season could be restricted to females.

- Transport of gametes: useful when male and female gametes are collected in different locations. This enables also the introduction of genes from the wild into hatchery stocks.
- Avoiding aging of sperm: the senescence of sperm during the course of the spawning season has been reported for many fish species and results in a decrease of milt quality (Rana, 1995a). Cryopreservation allows the collection of sperm when it has the highest quality.
- Experimental programs: for genetic studies, in comparing the breeding performances of successive generations in the same experiment and for experiments where the use of identical sperm samples is necessary over an extended period e.g study of short term storage of ova.
- Conserving genetic variability in domesticated populations: the use of a limited number of breeders leads to a reduction of heterozygosity. The cryopreserved semen of selected strains or genetically improved populations can be introduced in domesticated stocks e.g. the sperm of sex reversed gynogenetic hirame female *Paralichthys olivaceus* Temminck & Schlegel (Tabata and Mizuta, 1997). Gene banks of cryopreserved semen can also be used to maintain genetic diversity of fish populations that are endangered and protect against inbreeding. In protogynous hermaphrodite species such as Black grouper (*Epinephelus malabaricus* Bloch and Schneider), sperm can only be collected in 5 to 10 years old animals (Gwo, 1993). As a consequence, success in breeding is greatly enhanced by the use of frozen sperm.

If effective cryopreservation can be developed as good as in livestock cryopreservation, then the fish propragation of Thailand would be advanced in the future. The objective of this study was to compare the effective of short and long storage or cryopreservation of sperm in *C. microlepis*.

## MATERIALS AND METHODS

### *Sperm collection*

Milt was collected from mature male broodfish, reared in earthen-pond. The quality of milt was checked according to its motility. Only milt with motility more than 80% was used. The milt was transported to the laboratory (15 minutes) in a cooler at ~ 4 °C.

*Short – storage (4-9 °C)*

**Extender of Milt**

Extender solution should be compatible with seminal plasma or blood of candidate species (reduce activity of sperm before freezing) i.e., 280-300 mOsm/kg for freshwater species and 200-300 mOsm/kg for marine species. In this study, milt was diluted 1:5 with Modified Cortland's solution (MC: NaCl= 0.65 g, KCl= 0.3 g, $CaCl_2$ = 0.03 g, $NaHCO_3$ = 0.02 g at 100 ml, pH = 7.9). The extended milt was immediately drawn in to 1 ml tube and kept in refrigerator (4-9 °**C**).

**Experimental design**

The experiment was design with 2 treatments as followed:

- Treatment 1 fresh milt
- Treatment 2 fresh milt + Modified Cortland's
- Post fertility of Short – storage Milt

To evaluate the fertility of sperm, milt from all treatment groups were used to fertilize the eggs from a single female. Each cryo-tube was added to ~ 200 eggs. The eggs were incubation in plastic tank (size 10 l). Fertilization was estimated from the percentage of late gastrula.

*Long-storage or cryopreservation (-196 °C)*

**Cryoprotectant**

Function to protect cell destruction during freezing and thawing. There are 2 groups of cryoprotectants as follow:

1. Permeating (intracellular) cryoprotectants; these can permeate into cells for example DMSO, glycerol, MeOH, 1, 2 propanedio, and ethylene glycol

2. Nonpermeating (Extracellular) cryoprotectants; these can not permeate into cells. They are include: sugar (sucrose), polymers, starch, Polyvinylpyrrolidone (PVP), protein (egg-yolk, skim milk)

The milt was diluted 1:5 with 10 % milk + 2.5 % glucose + 10 % methanol equilibration period at 4°C. The extended milt was immediately drawn in 1 ml cryo-tube. The cryo-tubes were sealed and freezing at the rate of -2.95 °C per min. on cane #1 (about 19 cm above the liquid nitrogen surface) for 20 minutes before plunge into liquid nitrogen.

**Experimental design**

The experiment was designed randomly with 2 treatments as followed:

- -Treatment 1 fresh milt

- -Treatment 2 fresh milt + 10 % milk + 2.5 % glucose + 10 % methanol

**Post fertility of cryopreserved Milt**

To evaluate the fertility of frozen sperm, milt from all treatment groups were thawed on the same day and were used to fertilize the eggs from a single female. The cryo-tubes were thawed in water bath at ~70°**C**. The milt was added to eggs just as the thawing milt was going through the transition from solid to liquid. Each cryo-tube was added to ~ 200 eggs. The eggs were incubation in plastic tank (size 10 l). Fertilization was estimated from the percentage of late gastrula.

*Statistical analysis*

Differences in fertilization, hatching and survival rate relative to the time of freezing were test by use of a paired t-test. The null hypothesis was rejected for test of significance when $p<0.05$

RESULTS

*Short–storage*

The milt was preserved in a short–storage (4-9°C) by either keeping concentrated milt or diluted milt with Modified Cortland's. The short–storage milt was diluted 1: 5 with Modified Cortland solution can be kept for 48 hrs. Efficiency of the milt after preservations was tested by sperm motility, fertilization rate, hatching rate and survival rate were 30, 70.88±5.03, 75.59±4.50 and 77.87 7.92 %, respectively.

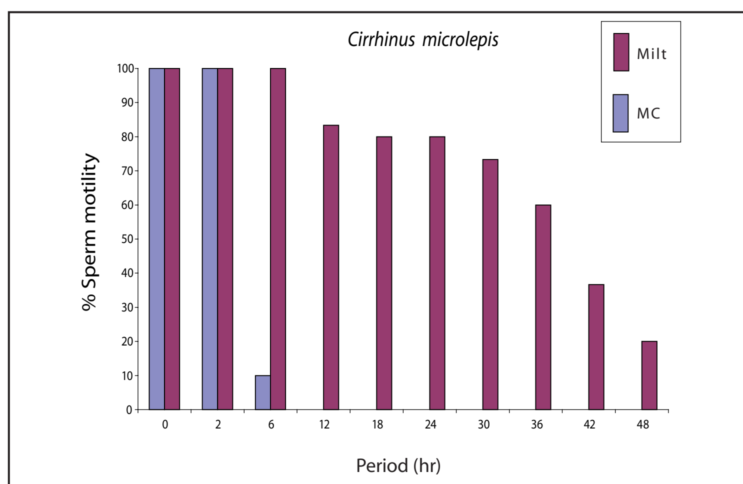
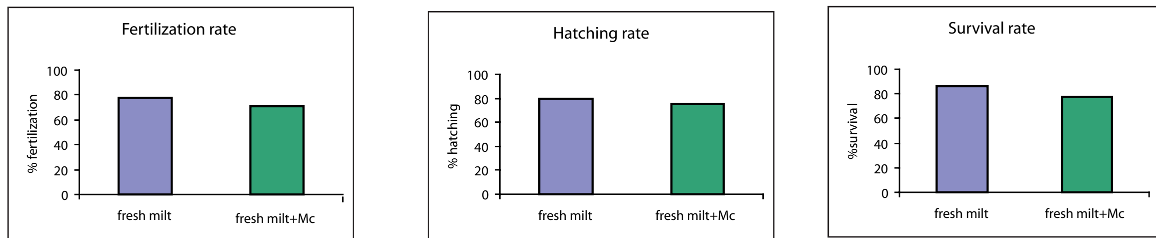



Figure 1. Differences in sperm motility rates in modified Cortland solution (MC) and milt

Percentage of fertilization, hatching and survival rate in fresh milt and diluted milt was not significantly different ($p>0.05$)




Figures 2-4. Comparisons of the effectiveness of fresh milt and fresh milt+MC of the *Cirrhinus microlepis* by monitoring the fertilization rate after kept in refrigerator (4-9 °C) for 48 hrs (left), the hatching rate (centre), and the survival rate (right)

*Long-storage*

The milt was preserved in a long-storage or cryopreservation at -196°C. The milt was diluted 1:5 with 10 % milk+2.5 % glucose+10 % methanol and equilibrated at 4°C for 15 minutes. Then diluted milt was freezing at a rate of -2.95°C per min or cane #1 for 20 minutes before plunge into liquid nitrogen. Efficiency of the milt after preservations at 2 hours was tested by sperm motility, fertilization rate, hatching rate and survival rate were 30, 62.66±17.59, 67.04±25.85 and 63.66±20.10 %, respectively.

Percentage of fertilization, hatching and survival rate in fresh milt and milt was diluted 1:5 with 10 % milk+2.5 % glucose+10 % methanol was not significantly different ($p>0.05$).

Final temperature diluted milt to freezing rate at Cane#1 Cane#2 and Cane#3 for 20 minutes

- Cane #1 (19 cm from surface): -47.1°C
- Cane #2 (14.5 cm from surface): -116.6°C
- Cane #3 (9.5 cm from surface): -190.7°C

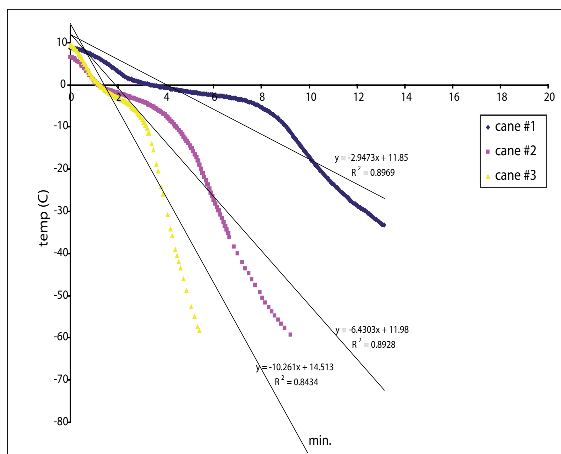
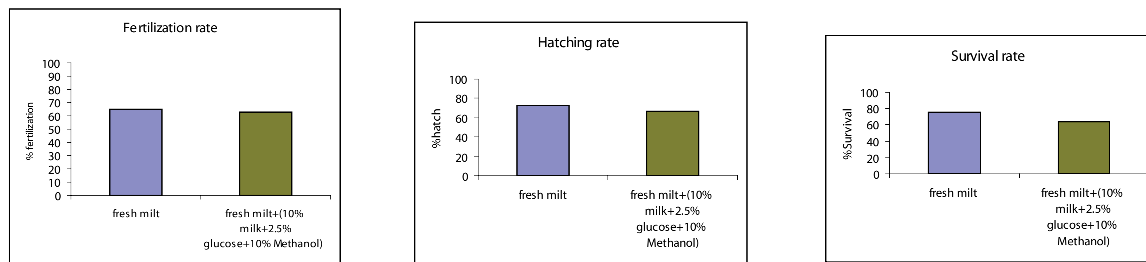



Figure 5 Diluted milt to freezing rate at cane #1, cane #2 and cane #3 before plunge into liquid nitrogen.




Figures 6-7. Comparisons of the effectiveness of fresh milt and diluted milt in 10 % milk + 2.5 % glucose + 10 % Methanol of the *Cirrhinus microlepis* by monitoring the fertilization rate after preservations at 2 hours (left), the hatching rate (centre), and the survival rate (right)

## CONCLUSION AND DISCUSSION

Preservation of fish milt begins with collecting milt by either striping or dissecting for testis. The milt is preserved in a short – storage (4-9°C) by either keeping concentrated milt or diluted milt with variety of solutions.For long-storage or cryopreservation at -196°C. The cryopreservation techniques for semen of freshwater fish are applicable for production purposes in aquaculture and for establishement of sperm banks. Coupled with insemination and short term storage techniques, cryopreservation will lead to an improvement of gamete management in freshwater fish species. The efficiency of the preserved milt decreases in the longer preservation period, different species, methods and diluted solutions. If effective cryopreservation can be developed as good as in livestock cryopreservation, then the fish propragation of Thailand would be advanced in the future.

From these results, for short – storage (4-9°C) it is recommended to use milt which was diluted 1:5 with Modified Cortland solution because milt which was diluted 1:5 with Modified Cortland solution can be kept (for 48 hrs) better than fresh milt (for 6 hrs).For cryopreservation at -196°C it is't recommended to use a technique for preservation of fish milt because these results not complete (reason by will be test sperm motility, fertilization rate, hatching rate and survival rate after kept in liquid nitrogen one year).

## ACKNOWLEDGEMENT

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