Bull. Fac. Fish. Hokkaido Univ.
**41**(4), 149-154. 1990.

# Cryopreservation of masu salmon sperm by the pellet method

Keisuke YAMANO**, Noboru KASAHARA***, Etsuro YAMAHA*
and Fumio YAMAZAKI*

## Abstract

Cryopreservation of masu salmon *Oncorhynchus masou* sperm using the pellet method was assessed in relation to three factors affecting fertilizatiton rates ; pellet volume, concentration of spermatozoa in the thawed suspension material, and the thawed suspension volume. Experiments were repeated three times with the same batch of eggs and cryopreserved sperm to examined the resulting degree of variations. One ml of milt was mixed with 3 ml of 0°C extender medium consisting of glucose and DMSO. A maximum fertilization rate of 85.2% was obtained on average by using 50 $\mu$l pellets. Fertilization rates increased in proportion to the concentration of thawed suspension material. The suspension volumes in the range of 5-20 ml had no effect under conditions using a 25-30°C thawing solution when attempting to fertilize approximately 100-120 eggs. Fertilization rates using cryopreserved sperm varied slightly when compared with those using fresh sperm. However, significant differences were rarely observed.

Masu salmon *Oncorhynchus masou* is an important fish of coastal commercial fisheries in northern Japan, and for recreational fishing in the inland waters.

In this species, almost all females, and a portion of males smoltificate into their sea-run form and migrate to the sea. However, other males remain in the rivers in a residual form[1,2].

The rates of maturation and smoltification are different in natural versus hatchery stockes[3]. Body size at smoltification varies for each, ranging from 8 to 16 cm in fork length. There are also wide growth or genetic variants in this species[1,4]. These wide variations in this species suggest the promising results of selective breeding to make more valuable hatchery stockes.

Establishment of gene banks of various natural stocks of masu salmon or to preserve the special sperm of males such as XX or YY males to control sex[5] will also be desirable in the future. We have attempted to develop reliable techniques of cryopreservation of masu salmon sperm to contribute to these objectives. As reported previously[6] using an extender medium of 0.3 M glucose with 10% DMSO as described by Stoss and Refstie[7] for rainbow trout gave better results when applied to chum salmon.

* *Laboratory of Embryology and Genetics, Faculty of Fisheries, Hokkaido University, Hakodate, Japan 041*
(北海道大学水産学部発生学遺伝学講座)
** *Present address : National Research Institute of Aquaculture, Tamaki, Watarai-gun, Mie 519-04*
(水産庁養殖研究所)
*** *Hokkaido Fish Hatchery*
(北海道立水産孵化場)

In this paper, the effects of sperm pellet volume, the concentration of spermatozoa in thawed suspension, and the volume of thawed suspension materials were examined using the same extender of glucose-DMSO medium in the cryopreservation of masu salmon sperm by the pellet method.

## Material and methods

Matured male masu salmon *Oncorhynchus masou* were obtained from the Mori Branch Hokkaido Fish Hatchery. Milt was stripped from 6-8 males and mixed for the experiments after checking the motility of spermatozoa. Ovulated eggs were taken by cutting the adbomen and then rinsing with physiological saline. The milt and eggs were cooled with ice during the experiments.

One ml of milt was mixed with 3 ml of cooled to 0°C extrender composed of 300 mM glucose with 10% DMSO[7]. Using a syringe despenser (Nichiryo Co. Ltd.), constant volumes of the diluted milt were dropped into preformed cavities on dry ice. The elapsed time, from dilution of milt to pellet on dry ice, was no longer than one minute. Frozen pellets were packed in plastic cases and immediately transferred to liquid nitrogen for storage.

Fertilization tests were carried out at 1-5 days after freezing. Pellets were thawed with 120 mM $NaHCO_3$ thawing solution at 25-30°C. Thawing was completed under agitation after 6-16 seconds. The thawed suspension material was immediately added to 15 g of eggs (about 100-120 eggs).

To examine the effect of pellet volume on the fertilization rate, the pellet volumes of 10, 25, 50, 100 and 200 $\mu$l were made and thawed with 20 ml of thawing solution at the same volume of one ml namely 100, 40, 20, 10 and 5 pallets at each pellet. Concentrations of the thawed sperm material were made by thawing various number of pellets in a constant volume of 20 ml 120 mM $NaHCO_3$ thawing solution.

Experiments were repeated three times with the same batch of eggs and the cryopreserved sperm. Eggs from the same batch were inseminated with fresh sperm to serve as a control group. Eggs were incubated in hatching baskets. The incubation temperatures ranged around 14-15°C. Ten to fourteen hours after insemination (8-32 cell stage), all eggs were fixed with Bouin solution to determine the fertilization rates.

## Results

*Effect of pellet volume*

The results are shown in Fig. 1. It was clear that the volume of one pellet effects the fertilization rate. The smallest pellets of 10 $\mu$l in the experiments gave the lowest fartilization rate, being average 37.5%. The fertilization rates increased with the volume of the pellets. The highest fertilization rate being 85.2% in aveage was obtained with 50 $\mu$l pellets. The rates decreased for larger volume pellets. For example 80.5% at 100 $\mu$l, and 76.6% at 200 $\mu$l, respectively. The optimum pellet volume was thus determined as 50 $\mu$l.

*Effect of concentration of spermatozoa in thawed suspension*

The relation between fertilization rate and volume of thawed pellets in 20 ml

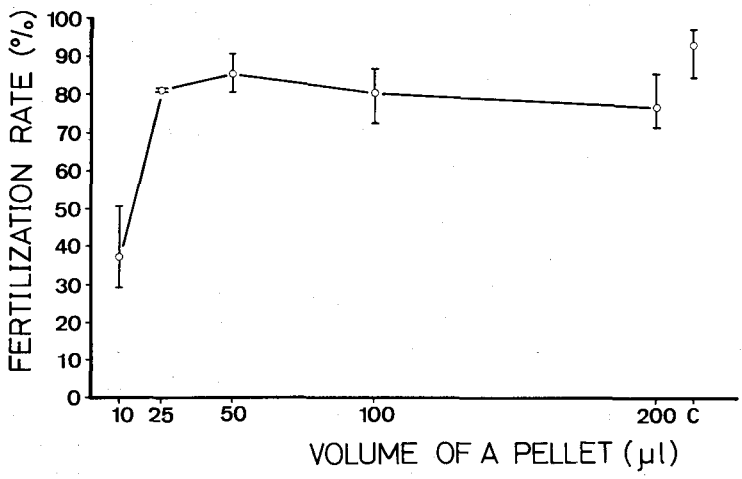


Fig. 1. Effect of volumes of one pellet on fertilization rate. Vertical bars indicate ranges. Total pellets in one ml were thawed with 20 ml of the thawing solution at 25-30°C to fertilize 15 g eggs (100-120 eggs). C: Control group with fresh sperm.

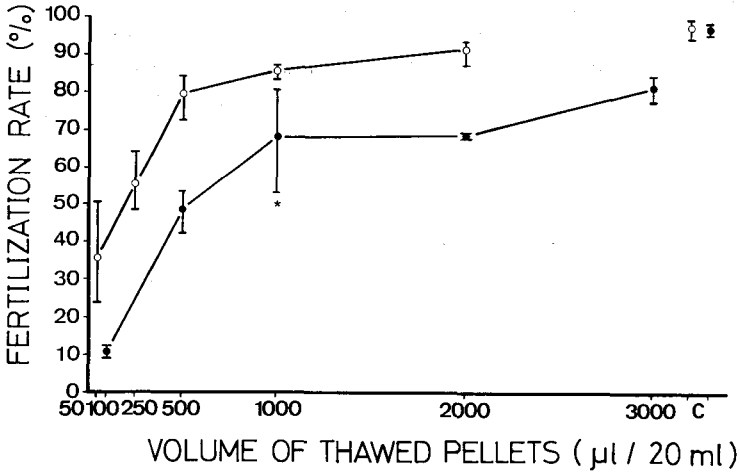


Fig. 2. Effect on fertilization rates by concentrations of spermatozoa in thawed suspension. Vertical bars indicate ranges. In one experiments, 20 ml of thawing solution at 25-30°C was used to thaw sperm pellets to be used in the fertilization of 15 g eggs (100-120 eggs). ○: Fifty $\mu$l pellet group, ●: Hundred $\mu$l pellet group. C: Control group with fresh sperm. *: Standard deviation was significantly ($P<0.05$) different from that of the control.

thawing solution is shown in Fig. 2 using 15 g eggs. The figure indicates that an increase in the volume of thawed pellets resulted in a steady increase in fertilization rates. The fertilization rates in 50 $\mu$l pellet groups were higher than the 100 $\mu$l pellet groups at the same concentration, these being 35.7% at 50 $\mu$l using a 50 $\mu$l

pellet group (one pellet/20 ml), 10.6% at 100 $\mu$l using a 100 $\mu$l pellet group (one pellet/20 ml), 91.3% at 2,000 $\mu$l using a 50 $\mu$l pellet hroup (40 pellets/20 ml) and 68.8% at 2,000 $\mu$l using a 100 $\mu$l pellet group (20 pellets/20 ml). The optimum concentration of pellets in 20 ml of thawing solution could not be determined in these experiments, although fertilization rates more than 80% were obtained when more than 100 $\mu$l of thawed pellets were used in 50 $\mu$l pellets.

*Effect of thawed suspension volumes*

Fifty and 100 $\mu$l pellets were thawed with 5, 10, 15, and 20 ml of thawing solution at the same concentration of 1 : 10 in 50 $\mu$l pellets, and 1.6 : 10 in 100 $\mu$l pellets. Five ml of thawing solution in which the final volumes of pellets were 5.5 ml using 50 $\mu$l pellets and 5.8 ml using 100 $\mu$l pellets were insufficient to cover 15 g eggs. Ten ml of thawing solution was sufficient to cover all of the eggs completely. No significant differences in fertilization rates were obtained in both groups of 50 $\mu$l and 100 $\mu$l pellets in any volume of thawing solutions tested in the experiments. A difference was however detected between control (99.4% in avarage) and 100 $\mu$l pellet groups (84.1% in average). The smallest volume (5.5 ml) of thawed suspension material was enough to fertilize 15 g eggs.

*Variations in fertilization rates*

The average standard deviation of fertilization rates for the experimental groups was 5.8. The value was greater than that of control groups of 3.1 on average. But significant differences in standard deviations between two groups were noted only in one instance of 1,000 ml group at 100 $\mu$l pellets in Fig. 2.

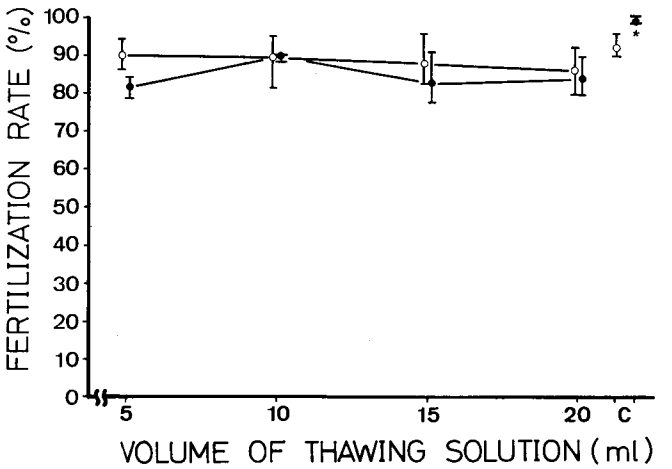


Fig. 3. Effect of a volume of thawed suspension material on fertilization rates. Vertical bars indicate ranges. The ratio of pellets to thawing solution volume was kept constant in 1 : 10 in 50 $\mu$l pellet group and 1.6 : 10 in 100 $\mu$l pellet group. The temperature of thawing solution was 25-30°C. In one experiment 15 g eggs (100-120 eggs) were used.
○ : Fifty $\mu$l pellet group, ● : Hundred $\mu$l pellet group. * : The average of the control group was significantly ($P<0.01$) different from those of 100 $\mu$l pellet group.

## Discussion

The present sudy showed that glucose-DMSO medium was useful as an extender in the cryopreservation of masu salmon sperm. This medium was first used in rainbow trout, Atlantic salmon, and sea trout and gave successful results in fertilization tests with frozen sperm[7]. This simple extender also gave higher fertilization rates in chum salmon sperm, when compared with three other defferent extenders[6].

Pellet volume is an important factor to obtain successful results because the volume greatly effects the freezing speed. The present study revealed that 50 $\mu$l volume of pellets was ideal, it gave the highest fertilization rate of 80.5% on average. The lowest rate was 37.5% on average using the 10 $\mu$l volume. Stoss[8] reported that during fast freezing, formation of intracellular ice injures the sperm cells and during slow freezing, increased concentrations of extracellular solutes expose the sperm cells to osmotic stress. There may be an optimum freezing speed in sperm cryopreservation for each species. This study could not determine the optimum freezing speed in masu salmon. However, it was observed that the freezing speed is not consistent for all areas of the pellests. Differences in freezing speeds at different regions of the pellets were clearly recognized, fast at the regions in direct contact with dry ice, and slow at free surface areas or central regions of the pellets. This difference in freezing speed in regions during pellet formation may increase with pellet volume. Ten $\mu$l pellets froze quickly because a large part of it was in direct contact with the dry ice. The resulting high freezing speed probably caused its low fertilization rate. Fifty $\mu$l pellets may consist of both fast and optimum freezing speed regions, thus resulting in its higher fertilization rate. Two hundred $\mu$l pellets may consist of fast, optimum and slow freezing speed regions. This might be the reason behind lower fertilization rates observed in the 200 $\mu$l pellets versus the 50 $\mu$l volumes.

Legendre and Billard[9] reported that the 1/100 dilution of frozen rainbow trout sperm always gave significantly higher fertilization rates than the 1/1,000 dilution. Stoss and Holts[10] examined the effect of sperm density in the thawing solution by making thawed suspensions of 1, 2, 5, 10, 20 and 50 pellets (each 50 $\mu$l) in 5 ml of thawing solusion at 9°C using rainbow trout sperm. They used 114-144 eggs in one experiment. As a result, a rapid increase in fertilization rates occurred between 50 $\mu$l to 150 $\mu$l sperm volumes, nearly equal rate occurred between the 250 $\mu$l to 1,000 $\mu$l volumes, and a considerable decrease for the 2,500 $\mu$l volume were reported. In the present study, fertilization rates increased in proportion to the sperm volume in the thawing solution. Also, a 2,000 $\mu$l or 3,000 $\mu$l pellet volume in the thawing solution gave still higher fertilization rates. This disagreement with the results of Stoss and Holts[10] might probably be caused by differences in temperature and volume of the thawing solution. Twenty ml of 25-30°C thawing solution was sufficient to thaw the pellets quick enough to fertilize the eggs. However, when less than 20 ml of thawing solution was used, higher fertilization rates of more than 80% were obtained from the present study. Five ml thawing solution was still sufficient to fertilize approximately 100-120 eggs when using a thawing solution at 25-30°C.

From the present study we recommend 20-40 pellets (each 50 $\mu$l) in 20 ml of thawing solution at 25-30°C to get consistently higher fertilization ratios of more than 80% in masu salmon. The experimental group however showed slightly greater variation and lower fertilization rates than control groups, although the

differences were rarely significant.

To minimize these differences between control and experimental groups, sperm cell injury caused by the cycle of freezing and thawing should be eliminated by developing improved extenders or pelleting methods. It is interesting to note that DMSO and glycerol have different cryoprotective effects on Atlantic salmon sperm[11].

## Acknowledgement

We wish to express our cordial thanks to Dr. Akira Goto and graduate students of Laboratory of Embryology and Genetics and staffs of Mori Branch Hokkaido Fish Hatchery, who helped us during the course of the present studies.

## References

1) Kubo, T. (1980). Studies on the life history of the "masu" salmon (*Oncorhynchus masou*) in Hokkaido. *Sci. Rep. Hokkaido Salmon Hatchery* **34**, 1-95. (In Japanese).
2) Utoh, H. (1981). Life history and ecological divergence of masu salmon, *Oncorhynchus masou* Brevoort, with special reference to a period of fluvial life. Doctoral Thesis, Faculty of Fisheries Hokkaido Univ. 1-288. (In Japanese).
3) Kato, T. (1982). Relationship between maturation and smoltfication in one year old male masu salmon reared in ponds. *Masu Salmon Research, Hokkaido Salmon Hatchery* **2**, 4-11. (In Japanese).
4) Okazaki, T. (1989). Population structure of masu salmon during their wintering migration along the coastal waters of northern Japan. *Physiol. Ecol. Japan*. Spec. Vol. 1, 359-369.
5) Okada, H. (1987). Artificial sex control and sterility in rainbow trout. *Fish Genet. Breed. Sci.* (*Suisan Ikusyu*) **12**, 1-16. (In Japanese).
6) Yamano, K. and Yamazaki, F. (1987). Cryopreservation of chum salmon sperm by the pellet method. *Fish Genet. Breed. Sci.* (*Suisan Ikusyu*) **12**, 45-50. (In Japanese).
7) Stoss, J. and Refstie, T. (1983). Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture* **30**, 229-236.
8) Stoss, J. (1983). Fish gamete preservation and spermatozoan physiology, in *Fish Physiology* ed. by W.S. Hoar, D.J. Randoll and E.M. Donaldson. Vol. **9**, Part B, Acad. Press. New York. 305-350.
9) Legendre, M. and Billard. R. (1980). Cryopreservation of rainbow trout sperm by deep-freezing. *Nutr. Repr. Devlop.* **20**, 1859-1869.
10) Stoss, J. and Holts, W. (1981). Cryopreservatjon of rainbow trout (*Salmo gairdneri*) sperm. 1. Effect of thawing solution, sperm density and interval between thawing and insemination. *Aquaculture* **22**, 97-104.
11) Yoo, B.Y., Ryan, M.A. and Wiggs, A.J. (1987). Loss of protein from spermatozoa of Atlantic salmon (*Salmo salar* L.) because of cryopreservation. *Can. J. Zool.* **65**, 9-13.