

# Cryopreservation of zebra fish spermatozoa using methanol<sup>1</sup>

BRIAN HARVEY,<sup>2</sup> R. NORMAN KELLEY, AND M. J. ASHWOOD-SMITH

Department of Biology, University of Victoria, P.O. Box 1700, Victoria, B.C., Canada V8W 2Y2

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We describe a method for cryopreservation of milt from individual *Brachydanio rerio* using methanol and powdered milk as cryoprotectants. Motility was positively correlated with hatching, which averaged  $51 \pm 35.6\%$  in a typical experiment. Variability in motility and hatching was not correlated with sperm volume or age of the fish, and is believed to be due to differences in sperm quality between individuals, as well as technical constraints imposed by the short duration of motility in thawed spermatozoa.

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On trouvera ici la description d'une méthode de cryoconservation de la laitance de *Brachydanio rerio*, méthode dans laquelle le méthanol et le lait en poudre servent d'agents de conservation. La motilité est en corrélation positive avec l'éclosion ( $51 \pm 35,6\%$  en moyenne au cours d'une expérience typique). Les variations de la motilité et de l'éclosion ne sont pas reliées au volume de sperme ou à l'âge du poisson et elles sont probablement attribuables à des différences dans la qualité du sperme d'un individu à l'autre de même qu'aux contraintes expérimentales imposées par la courte durée de la motilité des spermatozoïdes décongelés.

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## Introduction

The zebra fish (*Brachydanio rerio*) has become a popular laboratory animal by virtue of its ready availability, hardiness, and prolific breeding habits. Laale (1977) has reviewed a wealth of published material dealing with the physiology, biochemistry, and behaviour of this fish, and the production of homozygous clones by Streisinger and co-workers (1981) has facilitated the study of behavioural mutants.

In long-term studies with the zebra fish, such as those involving the isolation of mutants and the assessment of environmental effects, workers eventually confront a space problem which could readily be solved by storing the gametes of selected individuals. The method reported here was developed in order to streamline the artificial fertilizations necessary in our own experiments on the effects of low temperature on ova and embryos.

There is a large and confused literature on the cryopreservation of fish spermatozoa. Most work has been done on cold-water and marine forms, with salmonids by far the best represented (see Scott and Baynes (1980) for review). Dimethyl sulfoxide (DMSO) and glycerol predominate as cryoprotectants; success in fertilization with cryopreserved sperm is difficult to assess because of a lack of standardization in reporting results and varies widely. Few reports of

cryopreservation of sperm from tropical, freshwater fish have appeared (Withler 1980; Sin 1974). Yet storage of frozen sperm will become an important aquacultural tool (Harvey and Hoar 1979), and it was of additional interest to develop a method of potential use to fish farmers in the Third World.

## Material and methods

### Freezing of spermatozoa

Maintenance of zebra fish stocks has been described elsewhere (Harvey and Chamberlain 1982). Males were removed from stock tanks within 15 min of the start of the light cycle and anaesthetized individually in 0.09% 2-phenoxyethanol (Syndel Laboratories, Vancouver, B.C.) in dechlorinated water. The underside of the fish was blotted with absorbent tissue and the genital area scrupulously dried with filter paper; a 10- $\mu$ L glass capillary pipette cut to 100 mm was then held to the vent and milt was expressed by gentle tailward finger pressure. The average volume obtained in this way was 0.8  $\mu$ L.

Milt was immediately mixed with 5 volumes of the following freshly prepared diluent at room temperature (23°C): 9 mL fish ringer (Ginsburg 1963), 1 mL methanol (reagent grade), and 1.5 g skim milk powder (Carnation brand). Accurate mixing was facilitated by measuring the length (in millimetres) of the capillary tube occupied by milt and marking the tube at six times this value; diluent drawn up to this mark by capillarity was expelled and taken up, along with the accompanying milt, two to three times on a glass slide. It was important to avoid bubbles in the mixture because trapped air expanded violently during warming, usually causing loss of the sample. Immediately after mixing, the capillary tube was

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<sup>2</sup>Author to whom reprint requests should be addressed.

placed, unsealed, in a 12 × 100 mm pyrex culture tube within a 12-mL plastic conical centrifuge tube which was capped tightly and buried in solid CO<sub>2</sub>. This whole process, from milt collection to CO<sub>2</sub>, took about 3 min.

Freezing rate was measured using an IT-23 tissue-implantable thermocouple (Bailey Instruments, Saddle Brook, NJ) inserted in a diluent-containing capillary tube, and averaged 16°/min between 0°C and -35°C. After 20 min, by which time the temperature of the frozen milt had reached -79°C, tubes were uncapped while still in CO<sub>2</sub>, and the frozen samples were quickly transferred to liquid nitrogen. Storage time before fertilization was between 1 and 25 days.

#### Thawing and fertilization

Unfertilized eggs were obtained according to the method of Streisinger *et al.* (1981) except that 0.09% 2-phenoxyethanol was used as anaesthetic. Fertilizations were done in 60 × 15 mm plastic petri dishes (Falcon, Becton Dickinson Ltd., Oxnard, CA). Eggs were fertilized in groups of about 50; to minimize the delay between expulsion and addition of milt, females were placed in a holding bath of 0.045% 2-phenoxyethanol as each batch was fertilized. Each female provided 100–400 eggs.

Frozen sperm samples were thawed in air (390°C/min) to the point where ice just began to disappear, and then were expelled rapidly into 10 times their volume of 40% fish ringer in distilled water. After rapid mixing with a 50-μL micropipette, the activated sperm were added to the dry eggs, agitated gently, and flooded after 1 min with distilled water supplemented with 1 g/L sea salts (Marine Mix, Marine Enterprises Ltd., Towson, MD). This medium was changed twice on the day of fertilization and daily thereafter. Sperm motility was estimated subjectively by examining a droplet of the suspension used for fertilization at 400× magnification and noting the percentage actively moving, speed of movement, and density. Average elapsed time between addition of thawed milt to 40% fish ringer and microscopic examination of the sperm suspension was 30 s.

Unfrozen control milt was treated in two ways. In saline controls, eggs were fertilized with milt diluted 60× with 40% fish ringer. Diluent controls were treated exactly as described above under Freezing of spermatozoa with the omission of exposure to solid CO<sub>2</sub> and liquid nitrogen.

Fertilization and incubation of embryos were done in a thermostatically controlled room at 25°C. Daily records were kept of development and the results were expressed as the percentage of the starting number of eggs reaching stage 24 (retinal pigmentation) and hatching. Twenty males were used in the experiments reported.

### Results and discussion

The duration of forward movement of fish spermatozoa in the normal spawning medium varies widely, both between species and between individuals. The period of active motility is shortest in fishes spawning in fresh water, and usually lasts no longer than 2–3 min (Ginsberg 1972). Spermatozoa of the zebra fish maintain forward movement in distilled water for no more than 60 s, although dilution in fish ringer can extend this period for up to 1 h. Dilution of fish ringer to 40% for

sperm activation, as reported here, produced vigorously swimming sperm whose activity lasted long enough that microscopic estimates of motility could be made at the same time as fertilization. This is not usually possible with salmonids, in which the duration of active movement is so short that thawed milt is added to eggs in a slurry.

Average motility for 53 samples of cryopreserved sperm was 51 ± 18.2%. Variability was evident not only in the number motile but also in the speed of motility and in sperm density. Average sperm density was 4 × 10<sup>7</sup> to 8 × 10<sup>7</sup> spermatozoa mL<sup>-1</sup>. This variable appears unimportant within the limits employed here; varying the final dilution of milt between 5 and 100× had no effect on fertility.

Fertility was positively correlated with motility (Fig. 1 and Table 1); at *P* < 0.001, roughly 36% of the variability in hatching can be attributed to variation in motility. How closely hatching follows motility can be seen from the results of a single day's fertilizations, reproduced in Table 2; both are highly variable with hatching ranging from 10 to 103% of control values. The latter are shown in Table 3. The slightly lower figures for diluent controls (which are significantly different at *P* < 0.01 for hatching only) may reflect the longer time required for dilution (two steps rather than one) as much

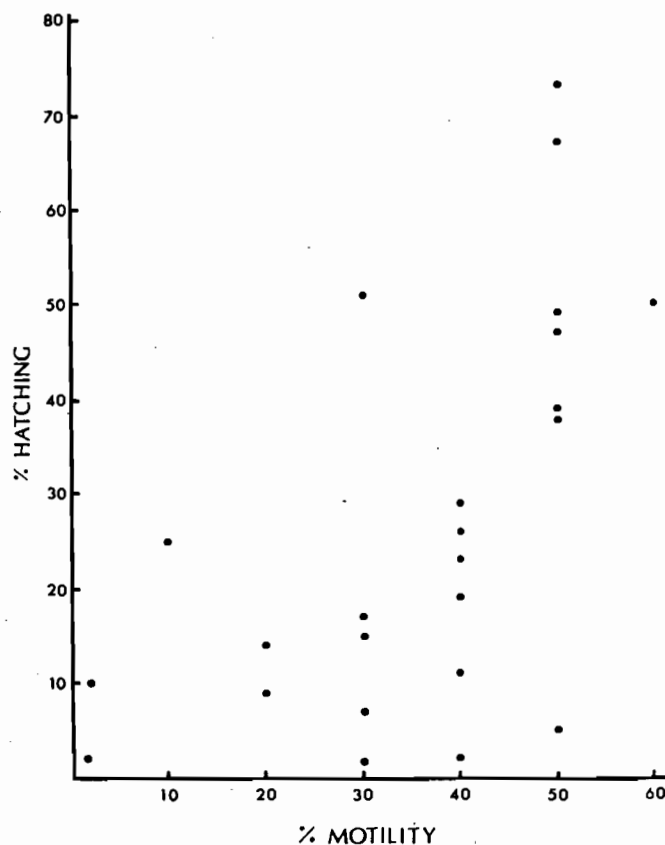


FIG. 1. Hatching versus motility of cryopreserved zebra fish sperm; *r* = 0.6; *n* = 25.

TABLE 1. Correlations between undiluted sperm volume, percent motility, age of fish, and percent hatching

Criterion (dependent variable)	Predictor (independent variable)	<i>r</i>	<i>P</i>
% hatching	% motility	0.604	<0.001
% motility	Sperm volume	0.007	<0.966
% motility	Elapsed time*	0.069	<0.626

\*Measured in days since the experiments were begun.

TABLE 2. A single day's fertilizations using cryopreserved sperm

% motility after thawing	♀	No. of eggs	% hatch*
50	1A	32	54
40	1B	36	16
40	1C	40	33
30	2A	60	10
50	2B	33	95
20	2C	44	13
50	2D	55	103
50	2E	35	69
60	2F	40	71

NOTE: Mean percent motility =  $43 \pm 12.3$ ; mean percent hatch  $51 \pm 35.6$ .

\*Percent hatch expressed relative to mean percent hatch for diluent controls (Table 3).

as any effect of the diluent on fertility. Fry from cryopreserved and control sperm appeared normal on hatching.

Methanol has been little used as a cryoprotectant, yet it is surprisingly nontoxic to a number of cell types. Ashwood-Smith and Lough (1975) demonstrated good protection of mammalian tissue-culture cells, and Morris (1981) found methanol superior to DMSO and glycerol in protecting *Euglena gracilis* and *Chlorella emersonii* to  $-196^{\circ}\text{C}$ . James (1980) has used high concentrations of this additive to freeze schistosomulae. The rapidity with which methanol enters and leaves cells (Harvey and Ashwood-Smith 1982; Ashwood-Smith 1980) confers a distinct advantage over protectants such as glycerol, where slower entry and exit from the cell demand longer equilibration times and more careful dilution procedures. Preliminary experiments in fact showed both DMSO and glycerol to be effective cryoprotectants with zebra fish sperm, but results using these compounds were often complicated by severe tail-to-tail agglutination. This problem was reduced by following a precedent from mammalian sperm-freezing (Polge 1980) and adding powdered milk as an extracellular cryoprotectant. Both fresh and powdered egg yolk, as well as lecithin, were ineffective; agglutination ceased to be a problem when methanol and milk were combined in the diluent.

TABLE 3. Control treatments for fertilization of zebra fish ova

Treatment	No. of eggs	% reaching stage 24	% hatching
Saline	287	$85 \pm 5.6$	$85 \pm 5.1$
Diluent	479	$76 \pm 6.2$	$71 \pm 5.2$

Cryopreservation success with fish spermatozoa is notoriously unpredictable, with much of the blame laid on the great variability between individual fish in semen quality and quantity (Scott and Baynes 1980). Our results fit this pattern and it seems worthwhile to attempt to explain the fluctuation in motility and hatching using cryopreserved zebra fish sperm. Table 1 shows that undiluted sperm volume is not correlated with motility, nor is the age of males over the course of the experiments. Sperm *quality*, as suggested by Billard and Breton (1976) may be important, and some of the variability in hatching is likely accounted for by differences in egg quality. A major source of variability, and one which is difficult to test, may be technical: active motility of thawed sperm is maximal 10–15 s after dilution and greatly diminished by 60 s. Thus there is little margin for error when samples as small as  $0.5 \mu\text{L}$  must be put through a complicated sequence of manipulations, and where premature activation of sperm can occur before or after freezing through inadequate drying of the fish, contact with condensation, or tardiness in applying diluted milt to eggs. Results would therefore be expected to be more consistent when larger volumes of milt are handled, and preliminary experiments using the same method to freeze the spermatozoa of *Tilapia mossambica* have in fact shown motility after thawing to be considerably higher for this fish.

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