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Short-and Long-term Cryopreservation of Sperm and Sperm Suspension of the Grouper, *Epinephelus malabaricus* (Bloch and Schneider)

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Abstract

Essential factors which affect the feasibility of cryopreserving sperm of the grouper, *Epinephelus malabaricus* (Bloch and Schneider), were determined by comparing percentage of motile spermatozoa, duration of storage life and fertilization rate of eggs when available. Fertility of sperm after thawing was maintained by using 10% dimethyl sulfoxide (DMSO) as a cryoprotectant with less than 10 minutes equilibration. An optimal ratio of 85% for sperm suspension from sliced testis or fresh milt diluted 20 times in Ringer solution for marine fish: 10% DMSO: 5% glucose is suggested. The addition of 15% Menezo Medium B2 INRA to the milt mixture resulted in the retention of higher progressive motility of cryopreserved sperm. The use of isopropanol with dry ice at -75°C or liquid nitrogen vapor at -100°C as freezing medium simplified the freezing procedure. Analysis of the motility of post-thawing sperm using the Hamilton-Thorn motility analyzer showed that the performance of one-and three-month old cryopreserved grouper spermatozoa was slightly poorer in terms of path velocity, progressive velocity, track speed and straightness. The use of microwaves in thawing frozen milt in polyethylene straws was found feasible and convenient with an optimum degree of power at 40% and conditional duration of 30-70 seconds. The post-thawing fertility of the spermatozoa was better when frozen immediately upon collection or right after transporting the material of raw milt or sperm suspension at 4°C for 1-2 hours. Fertility records of 78±18%, 83±3%, 95±0%, 75±32%, 13±1%, 95±0% and 66±12% were obtained for 1-, 17-, 41-, 54-, 128-, 278-and 291-day cryopreserved sperm (65.58-91.92% in controls) during the spawning seasons of 1990 and 1991.

Preliminary extension to a private hatchery in 1991 resulted in additional production of about 50 million fertilized eggs by using cryopreserved sperm of *E. malabaricus* eight times when fresh milt was not available in the field.

Introduction

The development of the marine finfish culture industry in Taiwan has accelerated remarkably in the past three years, mainly

because of setbacks in the prawn culture industry and land subsidence caused by overpumping of underground freshwater.

The culture of groupers is fast gaining economic importance. The total area devoted to grouper culture in Taiwan is about 240 ha. Annual production of cultured groupers has been relatively constant at about 1,000 tonnes valued at about US$6 million. Annual fry production by artificial means is about 2 and 3 million individuals in 1990 and 1991, respectively, for larval sources, while annual importations run at about 6 million wild fry. Thus, there is a great need to expand the artificial propagation technology to guarantee a good source of eggs and sperm.

In Taiwan, 32 species under the Genus *Epinephelus* have been reported to exist (Lee 1990). The grouper, *Epinephelus malabaricus* (Bloch and Schneider), erronously identified in several reports as *E. tauvina,* is the most widely cultured species and important economically. It is carnivorous, eurythermal and euryhaline. It is a highly favored seafood, especially in Taiwan, Hong Kong and Japan. However, supply of grouper fry has, thus far, been inadequate to meet demands. Improper capture methods, along with overfishing and destruction of its habitats have contributed to the depletion of the natural supply of fry.

To obtain a sufficient supply of fry, studies on the production of hatchery-produced fry have been carried out with considerable success in research centers such as the Tungkang Marine Laboratory, the Penghu and Tainan Branches of the Taiwan Fisheries Research Institute, and private hatcheries. However, several problems still need to be addressed, such as selection of suitable spawners, induction of sex reversal, determination of plasma vitellogen as indicator of oocyte maturation, availability of milt supply by means of low temperature or cryopreservation, as well as improvement of larval survival rates.

Survival of fish sperm can be maintained at 0-4°C by providing adequate air or oxygen to the milt sample (Billard 1981). Pioneering experiments on long-term preservation of fish spermatozoa at subzero temperatures were initiated by Hoyle and Idler (1968) and Graybill and Horton (1969). Since then, much work has been done on the cryogenic preservation of milt of freshwater fishes such as *Oreochromis mossambicus* Peters (Scott and Baynes 1980), *Cyprinus carpio* L. (Kurokura et al. 1984; Cognie et al. 1989), *Oreochromis* species and *Tilapia zillii* Gervais (Chao et al. 1987); and marine

fishes such as *Mugil cephalus* L. (Chao et al. 1975; Chao 1982), *Salmo salar* L. and *S. trutta* L. (Stoss and Refstie 1983) and *Morone saxatilis* Walbaum (Kerby 1983). Many other related studies were reviewed by Scott and Baynes (1980) and Harvey (1982). As far as typical protandric hermaphroditic fishes are concerned, the biological properties of *Acanthopagrus schlegeli* Bleeker sperm and its cryopreservation were investigated by Chao et al. (1986).

In preliminary observations of chilled and deep-frozen storage of grouper *E. tauvina* (Forsskål) sperm, motility was active after two-days storage, but no fertility was reported (Withler and Lim 1982). Our study aimed to investigate the properties of sperm of a typical protogynic hermaphroditic fish, *E. malabaricus*, and to develop a suitable extender and cryoprotective agent as well as a simplified procedure of cryopreserving its sperm for practical application.

Materials and Methods

Milt of grouper was obtained in two ways. Mature male groupers caught from the wild were dissected only in case of their mortality and their testes sliced and soaked in Ringer solution for marine fish (135 g NaCl, 0.06 g KCl, 0.02 g $NaHCO_3$, 0.025 g $CaCl_2$ and 0.035 g $MgCl_2$ in 100 ml H_2O) at 4°C before and during transport to the laboratory. Motility of mature spermatozoa free from testis was maintained in this suspension solution. Depending on where it was obtained, the milt was either airlifted from the Pescadores, a group of islands west of Taiwan, or from Kaohsiung in southwestern Taiwan, to the Taiwan Fisheries Research Institute headquarters in Keelung, northern Taiwan. The whole trip took slightly more than two hours. Fresh milt obtained from pond-reared spawners receiving or not receiving hormone treatment was the best material, although the collectible amount was usually less than 0.5 ml from each male. Thus, suction from the genital papilla using micro-haematocrit capillary tubes, followed by 10-20 times dilution, was done before the experiment.

To determine the effect of extender pH values on sperm motility, Ringer solutions for marine fish, with NaOH or $NaHPO_4$ added to obtain pH values of 7.0, 7.5, 8.0 and 8.5, were used. A series of $NaHCO_3$ concentrations, 0, 2.5, 5.0 and 10.0 mM was used

to investigate the effect of Na^+ ion on sperm motility. Antibiotics (streptomycin, neomycin and penicillin, with concentrations of 500, 1,000 and 1,500 ppm) were applied to compare their effect on prolonging the storage life of sperm at 4°C.

After comparing the effect of different concentrations of two cryoprotective agents, glycerol and dimethyl sulfoxide (DMSO), on the prefreezing motility of sperm, 10% DMSO was shown to be less toxic for 10-day exposure and was therefore used throughout the study during stepwise cooling and freezing. Milt mixture dispensed into polyethylene (PE) straws was equilibrated during dispensing at room temperature (usually less than 10 minutes) and then frozen using two protocols, namely, dry ice and liquid nitrogen (LN) vapor methods. In the dry ice method, 15 milt mixture samples dispensed into 0.5-ml PE straws were grouped into five and cooled at different rates in a mixture of isopropanol and dry ice at various subzero temperatures, namely, -10, -20 and -30°C for 5 minutes. The straws were then frozen to the final temperature of -75°C and immediately transferred to LN at -196°C. In the LN vapor method, 2 l LN were poured into a closed styrofoam box (40 cm in width x 25 cm in diameter x 25 cm in height) fitted with a PE net 2 cm above the LN surface. A digital thermometer (Suntex ST-52) inserted through the side of the styrofoam box was put right on top of the net when the PE straws containing milt mixture were frozen by LN vapor of known temperature (-100±10°C) for 5, 10, 15 and 20 minutes. The straws were then stored in an LN container.

To test the effect of Menezo Medium B2 INRA as an extender, the following experiment was done. In the control group, milt:DMSO:glucose at the ratio of 8.5:1:0.5 was prepared; in the experimental group, milt:DMSO:glucose:Menezo Medium B2 INRA was at the ratio of 8.5:1:0.5:1.5. Freezing was carried out by exposing straws to -100°C for 10 minutes before soaking in LN. Sperm motility right after mixing the cryoprotectant and extender, as well as after LN freezing and thawing, was compared using either a microscope or a Hamilton-Thorn Motility (HTM) analyzer. The following were analyzed and compared: detectable path velocity (the smoothed cell velocity and the five-point running average path velocity), progressive velocity (measured in a straight line from the beginning to the end of the track), track speed (computed by taking the total distance covered by a cell in its track, taking straight lines for each cell between the successive five points acquired, summing

the distances and dividing by the total elapsed time) and straightness (the ratio mean path velocity/mean progressive velocity, expressed as a percentage).

To evaluate the fertility of sperm thawed after freezing, newly stripped eggs of good quality were obtained from hormone-induced mature spawners. Straws containing frozen sperm were brought out of the LN container after different periods of storage and immediately soaked in room temperature water. Thawed sperm was poured onto the eggs and artificial propagation was carried out as usual.

In addition to the traditional method of thawing frozen milt of fish by using ambient water bath, microwave heating techniques were also adopted in this study. First, microwave energy generated by a locally-made microwave oven at a series of efficiencies, namely, 20, 40, 60, 80 and 100%, was compared to determine the optimum efficiency by which frozen milt of grouper would thaw without breaking the straw or damaging the spermatozoa. Second, different durations of microwaves in thawing cryopreserved grouper sperm in PE straws were compared by exposing the straws to the efficiency of 40% for 30, 40, 50, 60, 70 and 80 seconds. After microwave thawing, the milt mixture was flushed out of the straw and diluted with seawater. Sperm motility was checked as soon as possible.

Results and Discussion

Mature Spawner and Sperm

E. malabaricus is a protogynic hermaphroditic grouper which generally starts sex reversal at a relatively old age, about four or more years old. In this experiment, reproductively-mature males weighing more than 25 kg each were used. Milt collected by stripping was extremely limited in volume, less than 0.5 ml in most cases. Sperm suspension obtained from sliced testes was further shown to be an ideal source of raw material (K. Suzuki, H. Kurokura and N.H. Chao, unpubl. data). Suction using a micro-haematocrit capillary tube maximized the use of the scarce milt and facilitated its transportation.

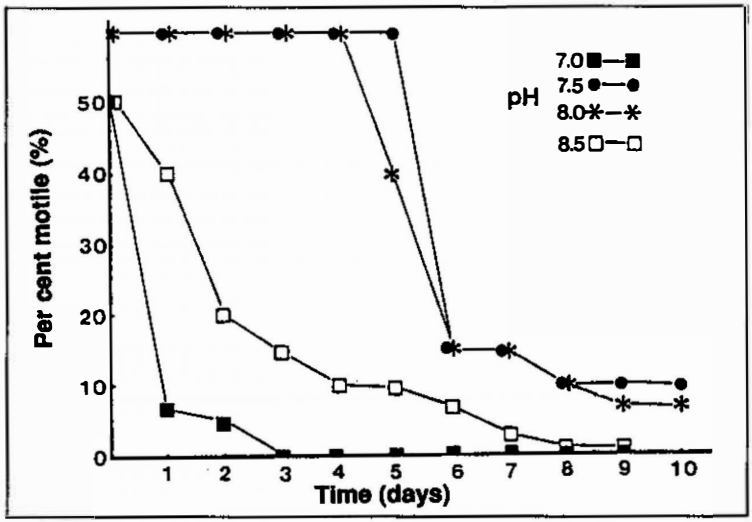

Fig. 1. Effect of pH value of extender on the motility of *E. malabaricus* sperm.

Factors Affecting Sperm Motility

The motile period of *E. malabaricus* sperm in seawater at room temperature usually lasted for more than 60 minutes. Percentage of motile sperm was 40-60%. Both parameters were used to evaluate the quality of the raw materials and to verify the effect of pH values, concentrations of Na^+, antibiotics, stepwise freezing on pre-freezing and thawed samples. The sperms were found much more active when pH values were at 7.5 or 8.0 than at 7.0 or 8.5 (Fig. 1). At pH 7.5 and 8.0, 60% of the sperm retained motility for four and five days, respectively. At pH 7.0 and 8.5, the initial contact of sperm with too low and too high pH inhibited motility, and percentage of motile spermatozoa dropped to 50% followed by 7, 5, 0% and 40, 20, 15% on days 1, 2 and 3, respectively. Billard (1984) indicated that optimal pH value is a major factor for sperms of *Dicentrarchus labrax* L. and *Sparus aurata* L. to express high spermatozoa motility. A similar result was obtained with *E. malabaricus* in our study. On the other hand, among 0, 2.0, 5.0 and 10.0 mM of $NaHCO_3$, the higher the concentration of Na^+, the shorter the maintainable motility was within a 9-day storage trial at 4°C (Fig. 2). $NaHCO_3$ at 10.0 mM with an osmolarity of 161-189 $mOsm \cdot kg^{-1}$ enhanced the motility of grouper sperm for six days. At

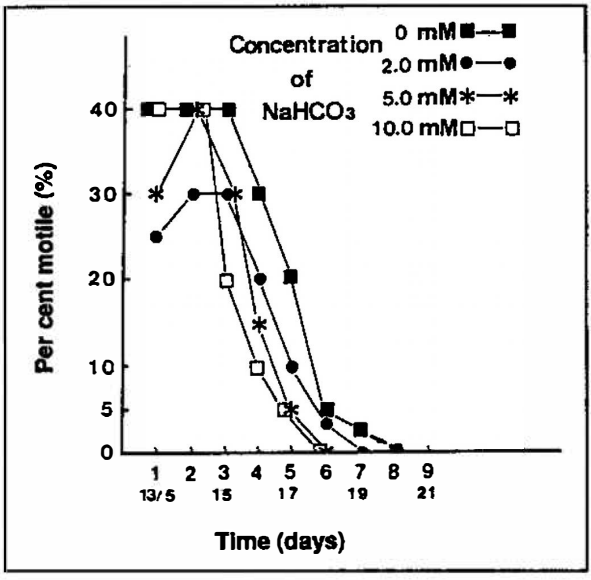

Fig. 2. Effect of $NaHCO_3$ at various concentrations on the motility of *E. malabaricus* sperm.

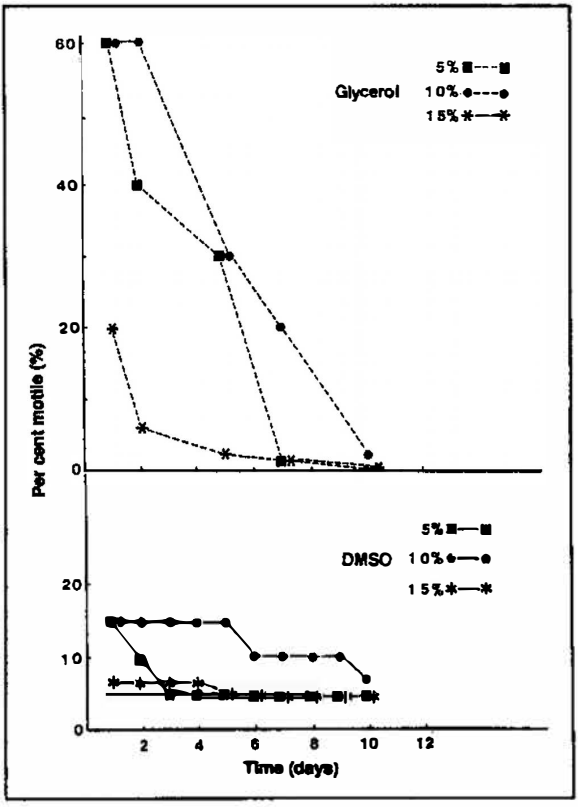

Fig. 3. Comparison of the effect of different concentrations of cryoprotective agents, glycerol and DMSO, on the prefreezing motility of *E. malabaricus* sperm.

5.0 and 2.0 mM, motility was less initially, but was maintained as long as that at 10.0 mM or longer.

Fig. 3 shows the effect of different concentrations of cryoprotective agents, glycerol and DMSO, on the prefreezing motility of *E. malabaricus* sperm. In both treatments, 10% was found most acceptable at continuous 10-day storage, although overall evaluation indicated that glycerol showed less inhibition than DMSO. In tests for post-freezing motility, 10% DMSO gave consistent good results.

Aside from the microscope, an HTM analyzer was also used, although only in the latter part of the study. The analyzer is accurate and more functional for analyzing and comparing sperm motility. Cosson et al. (1985) used stroboscopy to rapidly and quantitatively assess sperm motility. Future studies should compare the advantages and disadvantages of these machines in determining sperm motility.

Results of the trial on the addition of 15% Menezo Medium B2 INRA were encouraging. Frozen-thawed sperm motility improved, based on the analysis of their motility, progressive velocity and path velocity. In the ordinary diluent-cryoprotective agent group with seawater activation, 88% sperm had motility (47% rapid, 19% medium, 22% slow), 28% had progressive motility and path velocity was 112 μm/s before freezing. After freezing-thawing, 16% had motility (3% rapid, 13% medium, 0% slow), 3% had progressive motility and path velocity was 31 μm/s. In the Menezo Medium B2 INRA group with seawater activation, 86% sperm had motility (56% rapid, 14% medium, 16% slow), 24% had progressive motility and path velocity was 102 $\mu m \cdot sec.^{-1}$ before freezing. After freezing-thawing, 87% sperm had motility (27% rapid, 60% medium, 0% slow), 27% had progressive motility and path velocity was 31 $\mu m \cdot sec.^{-1}$. Usually, intensity of motility decreased with reduced osmotic pressure. Motility was induced when sperm was mixed with diluents having an osmotic pressure higher than the seminal plasma (Chambeyron and Zohar 1990). In our study, the intensity of motility was temporarily inhibited or significantly retained when Menezo Medium B2 INRA of an osmotic pressure (276-293 $mOsm \cdot kg^{-1}$) lower than the grouper's seminal plasma (330-350 $mOsm \cdot kg^{-1}$) was added. The composition of this patented and commercially-available cell culture medium is unnamed. Nevertheless, our study shows that it is promising for the long-term

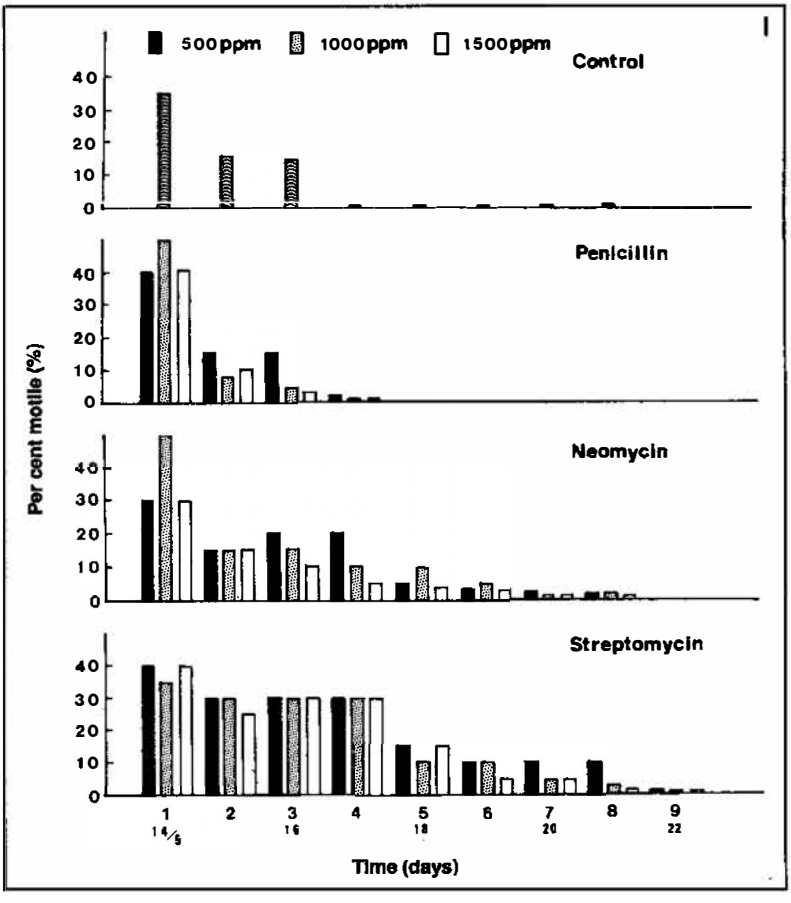

Fig. 4. Results of applying various antibiotics on maintaining the motility of *E. malabaricus* sperm.

preservation of sperm of grouper, a typical seawater fish, after it was reported to be effective for the cryopreservation of *C. carpio* (Cognie et al. 1989).

Short-term Preservation

Results of applying three antibiotics (streptomycin, neomycin and penicillin) indicated that streptomycin at either 500, 1,000 or 1,500 ppm gave better overall sperm motility than the others (Fig. 4). Functional motility higher than 10% lasted eight days in the group treated with 500 ppm streptomycin, five days in the group treated with neomycin and in the control, three days in the group

treated with penicillin. Streptomycin at 500 ppm is therefore recommended for short-term preservation at 4°C. Further studies are needed to test the preservation function by using other antibiotics to enhance and prolong sperm motility during the next spawning season.

Long-term Cryopreservation

Both dry ice and LN vapor methods were found equally applicable. The groups with various subzero temperatures of -10, -20 and -30°C resulted in 50.0, 55.0 and 55.0% motile sperm, respectively (60% in the control). Comparison of duration of exposing milt-DMSO-glucose mixture to LN vapor at -100°C on post-thawing fertility resulted in nonsignificant differences among 5, 10, 15, 20 minutes and control fresh sperm groups averaging 94.07, 89.51, 95.74, 94.52 and 93.82%, respectively.

Results of fertility tests of seven batches of cryopreserved sperm were encouraging. Although the 128-day group obtained a comparatively low fertility range of 13-1%, the 1-, 17-, 41-, 54-, 278- and 291-day groups had fertilities of 78±18%, 83±3%, 95±0%, 75±32%, 95±0% and 66±12%, respectively, which were not lower than the control group ranges of 66-92%.

The motility of long-term cryopreserved sperm was analyzed also using an HTM analyzer. Results are shown in Fig. 5. Taking fresh, one- and three-month cryopreserved sperm as samples, it was easy to read from the printout record that the total number of sperm with detectable path velocity, progressive velocity and track speed, as well as straightness over 50%, decreased and percentage of less motile spermatozoa increased with time. Using the HTM analyzer as a favorable instrument for comparison of motility under various combined conditions is worth further study.

The motilities of thawed sperm of several fish species such as *M. cephalus* (Chao 1982), *A. schlegeli* (Chao et al. 1986) and *T. zillii* (Chao et al. 1987), were found less durable when compared to fresh sperm. In our study, grouper sperm sometimes showed extraordinary durability of 88-120 minutes compared with 75 minutes in control after it was thawed from cryopreservation storage of up to three months. This indicated a big potential for field application. Hatchery operators can therefore thaw and mix different batches of sperm to get the optimal amount depending on

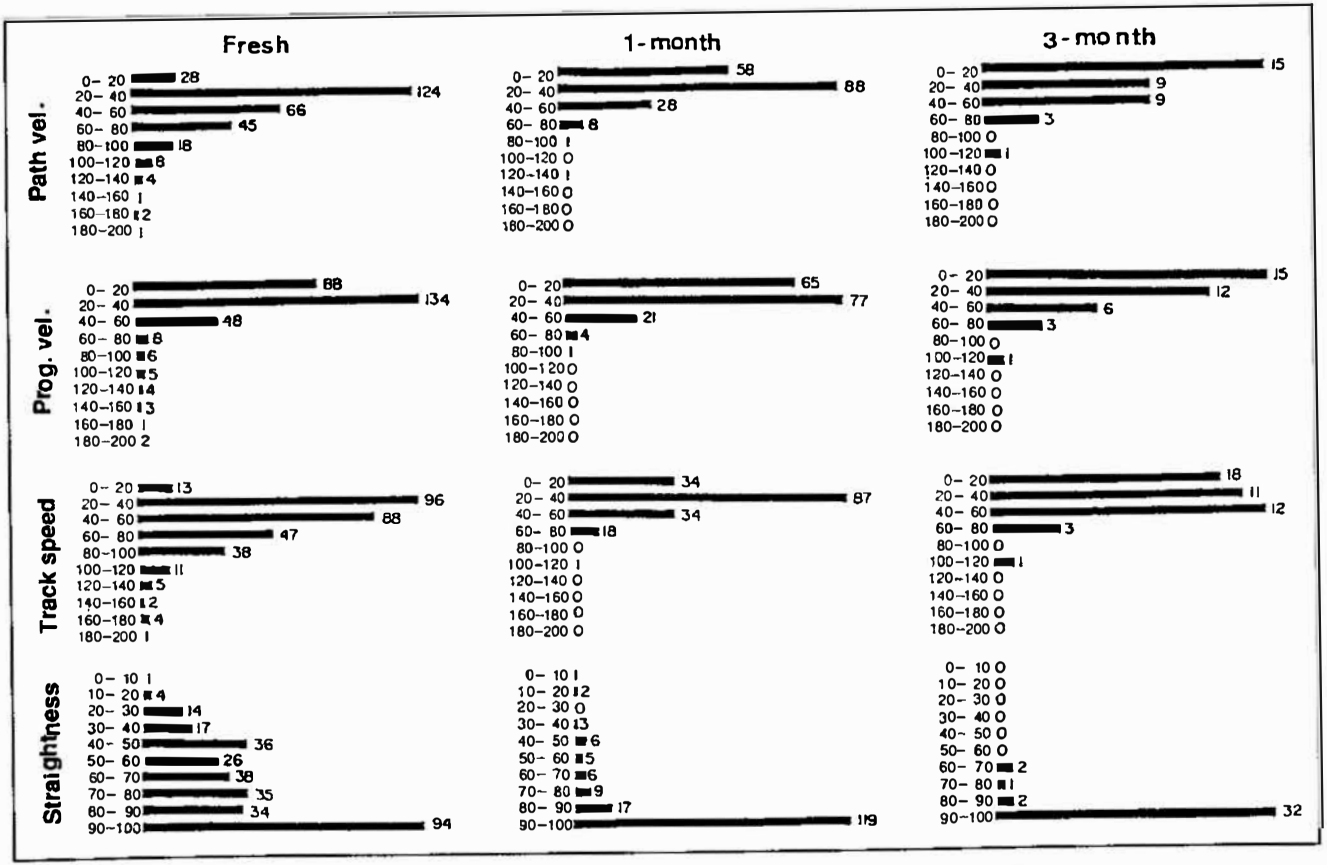

Fig. 5. Motility analysis and comparison of fresh, 1- and 3-month cryopreserved *E. malabaricus* sperm. Prog. vel. = progressive velocity.

the quantity of eggs obtained. Millions of eggs have a higher chance of being fertilized when such durable cryopreserved sperm is used.

The microwave thawing of frozen milt mixture in PE straws was found feasible and effective with the proper degree of power and exposure duration. In our case of adopting 0.5 ml orange, blue and green straws of high hardness (FHK, Japan), the optimum degrees of power and duration were 40% and 70 seconds, respectively. However, with light gray and light purple straws of lower hardness, the optimum microwave efficiency and duration were 40% and 30-35 seconds. Perhaps, the hardness of the straws had to do with the color material used in their manufacture. Cutting the straws quickly and immediately adding thawed sperm to the eggs helped to achieve the best utilization of the cryopreserved microwave-thawed sperm. Microwave ovens are gaining in popularity in local laboratories and hatcheries because they make thawing easier, quicker and neater. The preliminary results of our trial indicated no disadvantages except that overexposure might result in the bending and breaking of straws. Trials are therefore necessary. Frozen items can be thawed using microwaves, a method that has advantages over traditional methods. It is possible to thaw small or large quantities of cryopreserved fish sperm in a microwave oven. Results are repeatable, whether the temperature of the thawing solution or thawing bath varies with ambient temperature. As in Burdette et al. (1980), further studies concerning experimentally-effective designs and possible microwave damage on genetic characteristics of sperm should be considered. Thus, adopting the use of microwaves in thawing cryopreserved sperm will help cryobiologists and hatchery owners to achieve reliable and repeatable results.

The cryopreservation treatments and methods developed in this study are readily available and simple, thus packaging the technology for transfer to private hatcheries was easy. Field application of the treatments and methods is currently being undertaken. A local private hatchery in Taiwan, using the technology developed in this study, successfully produced an additional 50 million fertilized eggs by using cryopreserved sperm of *E. malabaricus* eight times during the spawning season of 1991 when fresh milt was not available from spawners in the field.

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