Bull. Inst. Zool., Academia Sinica, Monograph **16**: 263-283 (1991)

FISH SPERM CRYOPRESERVATION IN TAIWAN: TECHNOLOGY ADVANCEMENT AND EXTENSION EFFORTS

NAI-HSIEN CHAO

Department of Aquaculture, Taiwan Fisheries Research Institute, Keelung, Taiwan 202, Republic of China

N.-H. Chao (1991) Fish sperm cryopreservation in Taiwan: Technology advancement and extension efforts. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 263-283. It has been 20 years since the first study in Taiwan on cryopreservation of fish sperm of the grey mullet, *Mugil cephalus*. Several achievements have been made on the fish sperm cryopreservation, including technologies such as obtaining raw milt (stripping of spawners, getting sperm suspension from the sliced testis of recently killed spawners, or from inserting the microcapillary into the genital pore of scarce milt species), evaluating extender formulae (Ott and Horton extender 164, glucose, honey, cell culture medium), cryopreservative agents (glycerol, methanol or dimethylsulfoxide), freezing tools (isopropanol with dry ice, liquid nitrogen or vapor in Styrofoam box, programmable freezer or cooling system), thawing methods (water bath, warm thawing solution or microwave oven), the motility determination (microscopic reading or motility analyzer) and the fertility evaluation (timing, volume ratio and growth monitoring). These technologies are all efficient, convenient and economical, reflecting various stages of the development and studies in Taiwan. As a testimony to the quality of these technologies, researchers from Taiwan have collaborated with colleagues from the U.S.A., France, South Africa and Japan and published a series of papers on the sperm cryopreservation of the mullet, carp, catfish and grouper. Furthermore, these technologies have been disseminated and transferred through local training courses. Demonstration and assistance in facility have also been set up to the private sectors.

Key words: Sperm, Cryopreservation, Technology advancement, Extension, Taiwan.

The development of reproductive physiology in general and cryobiology in particular has directed a long term research in cryopreservation of fish gametes. An interesting area of cryobiology related researches

Table 1
Present status of research on cryopreservation of fish sperm in Taiwan

Species	Experiments on				Proof of motility after preservation		Fertility test in		Fertilization rate (%)[1]	
	Sperm characteristics	Extender selection	Optimal cryoprotectant	Freezing rate	Short term	Long term	Laboratory	Hatchery	Satisfactory frozen milt	Control fresh milt
Mugil cephalus	○[2]	○	○	○	○	○	○	○	64.9	23.6
									38.9	47.4
									48.9	38.4
Acanthopagrus schegeli	○	○	○	○	○	○	○	○	91.5	96.0
									77.4	62.3
									99.0	98.0
									71.4	84.5
Epinephelus malabaricus	○	○	○	○	○	○	○	○	95.7	93.8
									93.2	65.6
									85.0	65.6
									93.3	91.9
									84.6	91.9
Oreochromis aureus	○	○	○	○	○					
O. mossambicus	○	○	○	○	○	○				
O. niloticus	○	○	○	○	○					
O. niloticus×*O. aureus*	○	○	○	○	○	○	○		72.7	85.7
O. sp. (red tilapia)	○	○	○	○	○	○	○		93.4	90.0
Tilapia zillii	○	○	○	○		○	○			
Chanos chanos	○	○	○	○	○					
Siganus oramin	○	○	○	○	○	○				
Plecoglossus altivelis	○	○	○	○	○					
Lateolabrax japonicus	○	○	○	○	○					
Misgurnus anguillicaudatus	○	○	○	○						
Boleopthalmus chinensis	○	○	○		○					
Micropterus salmoides	○	○	○	○						

1. Only samples with good results are shown.
2. ○ work has been done in Taiwan.

aiming at facilitating the artificial propagation, cross-breeding, genetic improvement, predictable gene bank and chromosome manipulation are now established in Taiwan. These researches stem not only from the request of basic knowledge on life processes, but also from the pressing need to manipulate natural resources for the benefit of mankind.

It has been 20 years since the first study on the sperm cryopreservation of the mullet, *Mugil cephalus*, in Taiwan. Since then, some progresses have been made on the sperm cryopreservation in several local freshwater and marine fishes, such as the grey mullet (Chao *et al.*, 1975, 1982), tilapia (Chao *et al.*, 1987), black porgy (Chao *et al.*, 1986), milkfish (Chao and Liao, 1987a, 1987b), grouper (Chao *et al.*, 1992) and carp (Cognie *et al.*, 1989) (Table 1). The performance of sperm quality before and after pre-treatment, freezing and thawing are highly affected by a series of factors such as the milt-collecting season, methods of obtaining raw milt, methods of dispensing the sperms into containers, the temperature maintenance before pre-treatment, methods of transporting the sperms to certain destinations, the extender formula, the cryoprotective agents, freezing facilities, the protocol, cryopreservating conditions, the thawing method and the post thawing application. Successful fish sperm cryopreservation has been demonstrated by the technology, inter-country cooperation and technology extension.

The present paper aims to 1) review various aspects of the established technologies on the sperm cryopreservation of local fish species in Taiwan, 2) investigate the progress of promoting related technology, cooperation and extension, and 3) assess the prospects of their dissemination for field applications. The cryopreservation of fish sperm has passed its initial phase of collection and evaluation; some developed technologies are reliable for certain fish species. The present phase should be transfer and application of the technologies to the industry and related research fields.

VARIABLES RALATED TO THE QUALITY OF CRYOPRESERVED SPERM

Age of spawner and season for collection

The age at which the experimental finfish species reach maturity varies from one species to the other, *e.g.*, 10 months for tilapia, *Tilapia*

zilli; 1 to 1.5 years for black porgy, *Acanthopagrus schlegeli*, which is protandric; 2 to 3 years for grey mullet, *Mugil cephalus*; 5 to 8 years for milkfish, *Chanos chanos;* and at least 5 years for grouper, *Epinephelus malabaricus*, which is protogynic.

The aging of spermatozoa *in vivo* has been identified as a cause reducing the storage life of gametes at 4°C. Usually an early propagating season is not favorable for obtaining mature female gametes. It is, however, on ideal season for collecting male gametes of good quality. As soon as the peak of propagation season is over, eggs are still suitable but sperms are no longer of high quality, thus are not ideal for cryopreservation. Preservable durations of sperm decreased at the spawning season went by. Sperms collected in the early season can be stored at 4°C longer than in the late season. Similar results were found in European bass (*Dicentrarchus labrax*) (Billard, 1984). For the above mentioned species, seasons for the sperm collection are recommended as follows: February to July for the tilapia, December to February for the black porgy, December to January for the grey mullet, May and September to October for the milkfish, April to July for the grouper. In the hermaphroditic protandric black porgy cultured in pond, it is easy to collect large amounts of milt from the harvested fishes which are 1.5 to 2 years old. In addition to stripping the male spawner to obtain sperms, slicing testis of newly killed or dead fishes (within 10 hr) to obtain sperm suspension or inserting the microcapillary tube into the genital pore are the two recently developed methods for the grouper which has scarce milt (Chao *et al.*, 1992).

Maintennance of milt quality

- Collecting the sperm without the contamination from feces, blood, scales or external water.
- Holding the collected sperm at best condition by providing moisturized air for respiration; for example, 1/3 space of the container is filled with milt and 2/3 is filled with humidified air.
- Maintaining the collected milt at low metabolic rate by keeping the temperature to be as low as 4°C in the collection box in the field or during transportation. It was found that the collected sperm of postmortem *E. malabaricus* retained its viability for cryopreservation after

being stored at 4°C for several hours.

Extender

Ever since, livestock researchers have suggested that the use of egg yolk as a component of diluents for spermatozoa since it has favored the preservation of bull spermatozoa at 0°C or above. However, the egg yolk in protecting the experimental spermatozoa from fishes such as the grey mullet during freezing and thawing has not been appreciated although it is assumed to play a role in protecting the cell membrane with its lipoprotein fraction of the lower density characteristics.

In most of the early phases of the concerned studies, rather complicated formulae such as Ott and Horton extender # 164 (sodium citrate, glucose, lactose of various concentrations) were adopted to prepare diluents (Chao *et al.*, 1975). The effects of various concentrations of several important components on the sperm motility of the milt at the pre-freezing stage were investigated. However, the effort was not successful. Preparation was complicated; took much time and was not absolutely beneficial. It was found that the simplest formulations, such as 5% glucose, have generally been the most successful ones. Later on, the diluent of simple and single component, such as diluted 0.5% honey or glucose in Ringer solution was found well functional in both laboratory and field for the milkfish and the black porgy (Chao and Liao, 1987a). Milk diluted with Ringer solution and methanol was an ideal freezing medium for cryopreserving sperms of several tilapia species, including *Oreochromis aureus*, *O. mossambicus*, *Tilapia zilli*, *O. niloticus* × *O. aureus* hybrid and red tilapia (*Oreochromis* spp. hybrid). Without milk, the frozen-thawed sperm did not function because of the usually high stickness of the milt mixture in which spermatozoa were immobile. The composition of several diluents of extenders are shown in Table 2.

Most recently, it has been found that adding a cell culture medium, Menezo B2 Medium INRA (15%) to the glucose and Ringer solution was a very effective method of improving the pre- and post-freeizing sperm motility of groupers (Chao *et al.*, 1992). A similar observation was also recorded in common carp, *Cyprinus carpio*, by Cognie *et al.* (1989). More cell culture media are suggested to be tested for this purpose and on more finfish species.

As far as the dilution ratio is

Table 2
Composition of several diluents (extenders) (g/l)

Kind	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	Taps	Caps	Glucose	Yolk	Honey	Milk	Species
Marine fish Ringer	13.5	0.60	0.25	0.35	0.2	—	—	—	—	—	—	marine fish
Freshwater fish Ringer	7.5	0.2	0.20	—	0.2	—	—	—	—	—	—	freshwater fish
Taps	2.9	3.20	0.07	0.03	—	15 mmol	—	—	—	—	—	tilapia
Caps	2.9	3.20	0.07	0.03	—	—	15 mmol	—	—	—	—	tilapia
Milk in Ringer	7.5	0.20	0.20	—	0.2	—	—	—	—	—	150 ml	tilapia
V_2e	7.5	0.38	—	—	2.0	—	—	1.0	0.2	—	—	tilapia
V_2f	7.5	—	—	—	2.0	—	—	1.0	0.2	—	—	tilapia
Honey in Ringer	13.5	0.60	0.60	0.35	0.2	—	—	—	—	10 ml	—	black porgy; milkfish

Table 3
Comparison of cryopreservation procedures of fish sperm

Species	Extender	Cryoprotective agents	Dilution ratio	Equilibrium time (min)	Freezing medium
Grey mullet	Glu-Mar*	10% Glycerol 10% DMSO	1 : 1	15, 30 or 45	LN vapor followed by LN
Black porgy	Glu-Mar	20% Glycerol	1 : 1	10	Dry ice + isopropanol at −10°C followed by 2°C/min or LN vapor at −100°C for 10 min followed by LN
Grouper	Glu-Mar	10% DMSO	1 : 20	10	Dry ice+isopropanol at −20°C for 5 min followed by −70°C and LN; LN vapor at −100°C for 5-10 min followed by LN
Tilapia	Milk in freshwater fish saline	5% Methanol	1 : 1	0	Dry ice + isopropanol at −35°C followeed by 5°C/min and then LN
Milkfish	Milkfish serum/ honey	10% DMSO	1 : 4	10	LN vapor followed by LN
Spine foot fish	Glu-Mar	10% Methanol	1 : 1	10	
Ayu	V_2f	10% DMSO	1 : 1	5	
Japanese sea bass	Glu-Mar	20% Glycerol	1 : 1	10	

* Glu-Mar: Glucose in marine fish saline

concerned, 1 part milt: 1 part extender for sperms of the grey mullet, the black porgy and the tilapia; 1:4 for the milkfish; and 1:20 for the grouper were found acceptable.

Cryoprotective agents

Results from experiments indicated that the tonicity and composition of extender are not as important as the protectiveness of cryoprotectants. Cryoprotective agents (CPAs) provide cryoprotection to a labile enzyme, *i. e.*, catalase, and stabilize protein in unfrozen, aqueous solution. A wide variety of chemicals or compounds share this property, but only ethylene glycerol, methanol and dimethylsulfoxide (DMSO) were used in our studies.

CPAs can induce protein denaturation at higher temperature, thus are toxic to cellular systems. Cryoprotectants at high concentrations can prevent the ice formation and hence the concomitant freezing-thawing damage of cells. However, cryoprotectants at the same levels can be lethal to unfrozen cells. The toxicity of cryoprotective agents is a factor affecting the success of cryopreservation of sensitive cells such as sperm. For each experimental species, the effects of cryoprotective agents at various concentrations on pre-freezing motility of sperm have been studied.

In the absence of cryoprotective agents, very few spermatozoa can survive freezing. The addition of cryoprotective agents to the milt greatly extend the tolerance of spermatozoa to freezing when freezing rates are slow. It has been reported that the optimal cooling rate depends on the nature and concentrations of the cryoprotectants used. In the grey mullet and the black porgy, 5 to 15% glycerol functions well, while in tilapias 15% milk and 5% methanol as diluent-CPA mixture ensured satisfactory sperm motility and fertility. On the other hand, in the milkfish and the grouper, a high proportion of sperms in 10% DMSO retained their motility (Table 3).

In general, a majority of researchers working on the sperm-freezing of teleosts agreed in using either glycerol or DMSO with concentrations between 3.3 and 15% (v/v) as the cryoprotective agent (Guest *et al.*, 1976; Erdahl and Graham, 1980; Cognie *et al.*, 1989). In our studies, methanol was used only in tilapia (Chao *et al.*, 1987), as was in the zebra fish by Harvey *et al.* (1982). Both DMSO and glycerol at concentrations up to 20% (v/v)

would stimulate RNA synthesis (Ashwood-Smith, 1980). The stimulation was due to an increase in an initiation with the DNA template rather than the RNA polymerase.

Combination of small quantities of either DMSO or glycerol (intracellular agent, at a level without osmotic effects) with dextrose (extracellular agent) have remarkable effects on the cryopreservation of mammalian cells. Similar experiments should be done on cryopreserving fish sperm cells although a preliminary test in the black porgy failed to show any significant effects.

Equilibrium time

Some experiments have been carried out on varying the equilibrium time. However, in our study, sperm did not require a long period to reach equilibrium in DMSO or glycerol. Spermatozoa acquired an increased resistance to freezing and thawing if the milt was stored at temperature above 0°C for a period of time. Such treatment probably make the cell membrane hard, therefore, the spermatozoa enable to tolerate the thermal shock.

Both the motility of spermatozoa and the integrity of its shape were retained if the spermatozoa of the experimental fishes were incubated with DMSO or glycerol for some period of time, which is less than one hour in the grey mullet, or 10 min in the black porgy, tilapias and the grouper. Comparisons of various equilibrium time in the grouper sperms were not done. However, it was found that sperms were so hard that the equilibrium was delayed to bring the milt mixture to the freezing temperature up to 30 min. After freezing the thawing sperm cells still gave an acceptable fertility rate.

Freezing rate

The cooling rate is critical in cryopreservation. When a cell is cooled too rapidly, intracellular ice will form. To prevent the formation of intracellular ice, cells must be cooled slowly. For sperms of certain species in the presence of certain CPAs, there was a critical subzero temparature zone. Within this zone, cells would be exposed to increased concentrations of solutes and therefore should pass through this zone as quickly as possible. In contrast, it is within the same temperature range that the cells require a sufficient time to get rid of water so that ice will not form, in turn cells can stand cooling to the lower

temparture.

It is a reconciliation of these two factors which determine the optimal cooling rate in different CPAs. The temparature at which the spermatozoa could be plunged directly into liquid nitrogen (LN) without causing a further reduction in motility should be carefully determined. One of the consequences of the two-step freezing demonstrated that slow cooling to a very low temperature was essential to avoid the thermal shock. In contrast, very fast cooling rates could be tolerated only within certain temperature ranges. In the cases of the grey mullet, the black porgy and the grouper, two-step freezing, first to −100°C and then to −196°C, were applicable; while in the case of the carp, the cooling rate slightly differred and was 5°C/min from 2° to −7°C and 25°C/min from −7°C to −70°C; and in the case of tilapias, 20°C/min directly to −35°C and 5°C/min from −35°C to −75°C.

Freezing facilities

In freezing studies, various equipments were used, including an ordinary stainless cabinet equipped with an air sucker to squeeze air inward slowly or quickly for different freezing rates. Biological freezer BF5 sets in LN container provided different distances above LN surface, thus milt mixture samples in vial were frozen at different freezing rates. On the dry ice a series of tiny holes were formed by a heated iron bar. Large or small sized drops of milt mixture were applied to these holes, as a result freezing rates can not be determined. Programmable freezer was used because preset programs help to determine the most favorable freezing protocol. Nevertheless, the most acceptable and simplest device was a common Styrofoam box fitted with a polyethylene net which was 2 cm above LN. Then the sample straws were placed on the net when the vapor in the closed box reached a constant temperature of −90°C to −100°C. This device worked well for the simple freezing of spermd of grey mullet, the black porgy and the grouper, The device is also accessable to students and farmers who usually do not have expensive facilities or equipments.

Thawing rate

In early experiments, frozen milts were thawed in water bath at room temperature as there was little evidence that the speed of thawing was critical to the survival of

spermatozoa. In the other case of tilapia, 30:1 of water at 25°C to 29°C were sufficient to thaw the frozen straws in 30 sec. Wheeler and Thorgaard (1991) compared the combined effects of various water temperature with the thawing time designed as: 0°C, 15 min; 5°C, 90 sec; 10°C, 30 sec; 20°C, 20 sec; and 30°C, 15 sec. Only samples thawed at 0°C gave poor results. On the other hand, those thawed at 5°C to 30°C gave similar results. In the experiment on the sample of *E. malabaricus* (Chao *et al.*, 1992) microwave thawing was applied. Microwave efficiencies at 40%, 60%, 80% and 100% for 60 sec were compared and various durations, from 30 to 80 sec, were tested. The results indicated that efficiency at 40% was reasonable for thawing of 0.5 ml straws. However, duration of 30 to 35 sec was optimal for light colored straws with low hardness and 70 sec for dark colored straws with high hardness.

Motility determination

The microscope is used to study the pre-freezing and the post-thawing sperm motilities. A conventional standard to categorize sperm motility were listed in Table 4. Spermatozoa motility was assessed under a microscope (× 100) and non-motile, very weak, weak, moderate, strong and very strong motility were scored on a 0-5 scale (Guest *et al.*, 1976; Billard, 1984). The motility scores of the fresh sperms in all the marine fishes studied usually reached 5 while that of freshwater

Table 4
Categorization of fish sperm motility under microscope reading (after Guest *et al.*, 1976)

	Value	Observation
Maximum	5/+++++	All sperm moving vigorously; almost impossible to fix vision on any one spermatozoa
	4/++++	Some sperm moving slowly enough to see easily; most still whirling or swimming vigorously
	3/+++	Some sperm still; some sperm slowly swimming; some fast
	2/++	Very few fast swimmer; many still; some slow "lazy-looking" sperm.
	1/+	Only one or two sperm swimming in each field
Minimum	0/−	No sperm moving; Brownian movement the only detectable motion

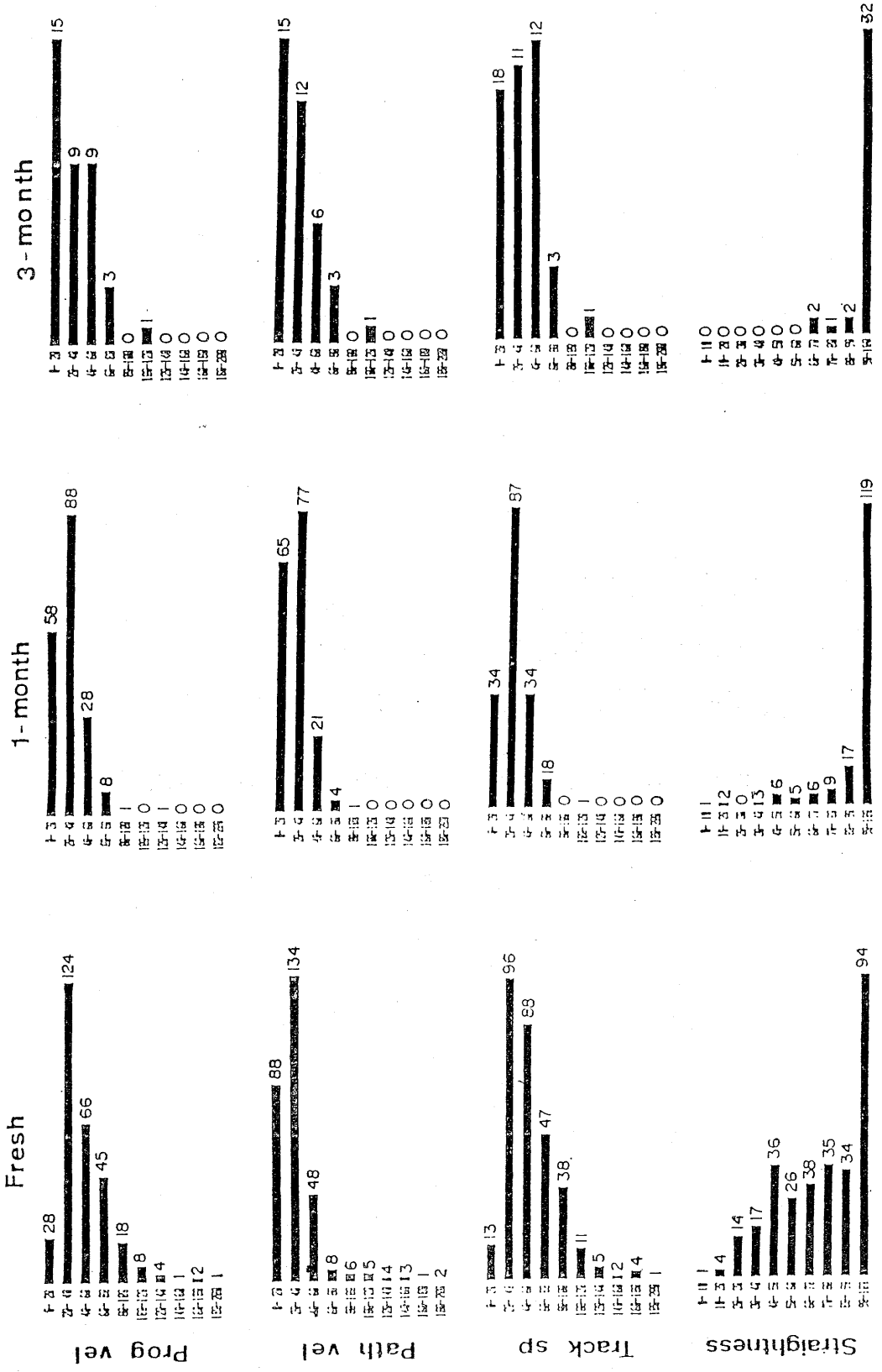

Fig. 1. Motility analysis and comparison of fresh, 1-month and 3-month cryopreserved *Epinephelus malabaricus* sperm.

tilapias were only 2 or 3, so that percentages of motile spermatozoa were sometimes used as another criterion.

Most recently, a motility analyzer (Hamilton Thorn) used for the medical research was adopted to analyse the sperm sample, which allows a big progress in motility determination. With the analyzer the following detailed criteria were able to be compared: 1) path velocity, 2) progressive velocity, 3) track speed, and 4) straightness. This instrument contains a fully automatic analytic sequence with internal optical image assembly, a microprocessor system for image analysis, and an internal printer, a color monitor in track velocity, progressive velocity and linearity and prints the numerical results. It tracks cells of various sizes between 1 to 120 microns using a cannula-made rectangular glass capillary. It has been used in mucus penetration of swim-up tests for the post-thaw milt. Fig. 1 shows the motility analysis and the comparisons of the fresh, 1-month and 3-month cryopreserved *E. malabaricus* sperms using the motility analyzer. More studies are needed to get familiar with other possible methods to determine the sperm motility.

Fertility evaluation

Fertility tests seemed to be less investigated. In marine fishes a good timing to obtain ripen eggs and optimal aeration during incubation were the most critical. The volume ratio of sperm to egg was hard to determine because the quality of post-thaw sperm varied from 1:1 to 1:6 seemed to be better than 1:13 if the frozen milt was prepared at 5 milt: 4 glucose (5%): 1 glycerol before freezing. In the tilapia, both 0.3:1 and 0.4:1 were adequate ratios to obtain a fertility over 90% when the frozen milt was prepard at a ratio of 50 milt: 15 milk: 10 methanol: 25 saline. In *E. malabaricus*, it was found that ratios ranging from 0.01 to 0.083:1 gave a satisfactory fertility over 80% when compared with the 92% in the control. However, in tilapias, which are mouth breeders, special facilities are needed in order to obtain the high fertility of post-thaw sperm. Three types of incubators were investigated and all were effective and efficient: 1) a shaking table with a speed of 26 shakes/min, 2) a zugerglass apparatus with upwelling water adapted from Mires (1974), and 3) round tanks with water circulation produced by an electric fan. Tables 5

Table 5
Results of thawing cryopreserved common carp sperm in 0.5 ml straws at 40% efficiency using microwave for various duration

Duration(s)	Straw color					
	White	Green	Orange	Blue	Purple	Red
30	10%+	2%+	10%+	−	−	−
20	10%+	80%++	80%++	80%++	70%++	80%++
10	10%+	80%++	80%++	70%++	−	80%++

++, good condition; +, moderate condition; −, bad condition.

Table 6
Results of fertility tests using cryopreserved and fresh grey mullet sperm to fertilize fresh eggs from the same female in each test

Test No. Year	Sperm preserved duration (days)	Cryoprotective agents	F.R. (%)	H.R. (%)	Remarks
I 1972	39	5% glucose+5% glycerol	15.6	61.3	in 3 liters
	39	5% glucose+5% DMSO	2.9	55.0	in 3 liters
	0		11.4	50.3	in 3 liters, control
II 1981	11	5% glucose+10% DMSO	28.6	62.2	in 3 liters
	11	5% glucose+10% DMSO	7.6	42.7	in 3 liters
	0		45.2	100.0	in 3 liters, control
III 1983	351	5% glucose+10% DMSO	38.2	92.0	in 3 liters
	0		35.8	93.5	in 3 liters, control

to 8 summarize the results of fertility tests with post-thaw sperms in the grey mullt, tilapias, the black porgy and the grouper.

Cooperation experience and outlook

When I talk about the cryobiological research, I prefer to say "We". Whatever accomplishments, I may have owed immeasurable gratitude to many project assistants, collegues and university professors who have worked with me in the laboratory. Thanks are also extend

Table 7
Results of fertility tests using cryopreserved and fresh tilapia sperm to fertilize fresh eggs from the same female in each test

Test no., Date	Sperm: Species	Sperm: Preserved duration (days)	Egg	F.R. (%)	H.R. (%)	Remark
I	*O. niloticus*×*O. aureus*	22	*O. honorum*	72.7	100	
16 July 1983	*O. honorum*	0	*O. honorum*	85.7	100	control
II	*O.* sp.	32	*O.* sp.	75.5	—	
12 September 1984	*O.* sp.	0	*O.* sp.	80.2	—	control
III	*O.* sp.	304	*O.* sp.	79.3	100	
8 July 1985	*O.* sp.	304	*O.* sp.	87.1	100	
	O. sp.	304	*O.* sp.	93.4	100	
	O. sp.	304	*O.* sp.	90.1	100	
	O. sp.	304	*O.* sp.	81.5	100	
	O. sp.	0	*O.* sp.	90.0	100	control
IV	*O. niloticus*	592	*O. niloticus*	90.2	97	
17 July 1985	*O. niloticus*	0	*O. niloticus*	94.6	95	control
V	*O.* sp.	455	*O.* sp.	30.0	100	
25 July 1985	*O. aureus*	8	*O.* sp.	90.5	100	
	O. niloticus	8	*O.* sp.	88.2	100	
	O. niloticus×*O. aureus*	335	*O.* sp.	75.8	92	
	O. sp.	0	*O.* sp.	50.0	90	control

Table 8
Results of fertility tests using cryopreserved and fresh grouper sperm to fertilize fresh eggs from the same female in each test

Test no. Date Time	Sperm: Preserved duration (days)	Sperm: Volume (ml)	Eggs (g)	F.R. (%)	Milt source and remarks
I	1	1.0	3	83.33	male 1
26 August 1990	1	0.5	3	67.39	male 1
1700 h	1	1.0	3	93.02	male 1
	1	0.5	3	8.60	male 1
	17	1.0	3	79.62	male 2
	17	0.5	3	85.00	male 2
	128	1.0	3	14.55	male 3
	128	0.5	3	12.12	male 3
	0	1.0	3	65.58	control
	0	0.5	300	80.95	control[1]

Table 8 Continued

Test no. Date Time	Sperm: Preserved duration (days)	Sperm: Volume (ml)	Eggs (g)	F.R. (%)	Milt source and remarks
II	1	1.0	3	80.00	male 1
26 August 1990	1	0.5	3	70.00	male 1
1830 h	1	1.0	3	60.71	male 1
	1	0.5	3	96.72	male 1
	0	0.1	3	91.76	control
III	17	2.5	15	83.61	male 2[2]
26 August 1990	1	1.5	9	91.43	male 1[3]
1830 h	0	0.1	3	91.76	control
IV	1	0.5	30	8.26	male 1[3]
26 August 1900	1	0.5	60	0.00	male 1[3]
1830 h	1	0.5	120	1.57	male 1[3]
	1	0.5	180	0.00	male 1[3]
	1	0.5	240	3.07	male 1[3]
	0	0.1	30	10.42	control
V	41	4.0	450	95.00	male 4[1]
30 May 1991	278	3.0	200	95.00	male 5[1]
VI	54	0.01	3	21.99	male 4
12 June 1991	54	0.02	3	48.75	male 4
1700 h	54	0.03	3	47.70	male 4
	54	0.04	3	69.30	male 4
	54	0.05	3	78.60	male 4
	54	0.10	3	82.03	male 4
	54	0.15	3	86.24	male 4
	54	0.20	3	83.55	male 4
	54	0.25	3	82.95	male 4
VII	291	0.01	3	49.54	male 5
12 June 1991	291	0.02	3	68.80	male 5
	291	0.03	3	67.48	male 5
	291	0.04	3	62.14	male 5
	291	0.05	3	69.22	male 5
	291	0.10	3	64.60	male 5
	291	0.15	3	72.54	male 5
	291	0.20	3	61.50	male 5
	291	0.25	3	78.68	male 5
	291	0.04	3	91.92	male 5

1. Mass fertilization done by aquafarmer in large-scale tank.
2. Medium scale using frozen-thawed sperm.
3. Trail to increase egg volume for fertilization using frozen-thawed sperm resulting in poor fertility due to poor quality of eggs.

Table 9
International cooperative studies involving R.O.C. on cryopreservation of teleost sperm

Country	Scientist	Year	Fish species	Major research items (key words)
U.S.A.	J.K. Sherman	1974	*Mugil cephalus*	Dilution, Cryoprotective agent
R.O.C.	N.H. Chao *et al.*			Prefreeze motility and fertility
South Africa	G.J. Steyn	1985	*Clarias gariepinus*	Extender, Cryoprotective agent
	J.H.J. van Vuren			Freezing rate, Thawing temperature
	H.J. Schoonbee			
R.O.C.	N.H. Chao *et al.*			
Japan	K. Suzuki	1989	*Epinephelus malabaricus*	Sperm suspension, Sliced testes
	H. Kurokura			
R.O.C.	N.H. Chao *et al.*			
France	P.F. Cognie	1989	*Cyprinus carpio*	Extender, Freezing rate
	R. Billard			
R.O.C.	N.H. Chao *et al.*			

to the collaboration of our friends from the private sector who provided good sources of raw milt of several experimental fishes and the opportunity to carry out fertility tests for cryopreserved fish sperms.

Taiwan Fisheries Research Institute (TFRI) has been cooperating with several foreign research institutions and universities on the cryopreservation of teleost sperms. Table 9 summarizes these international cooperative studies with scientists from USA, South Africa, Japan and France. In addition, TFRI cryobiologists also cooperate with local university professors in establishing different freezing protocols for cryopreserving sperms of the tilapias and the black porgy. Most recently, under a funding from the National Science Council of the Republic of China, a cooperative study on the cryopreservation of eggs and embryos of aquatic organisms with emphases on the development of an engineering model is under going to investigate the intracellular ice formation.

Further cooperative studies

among nations, institutions, universities, and scientists are expected to be more active on cryopreservation related basic problems, such as:

- Theoretical modelling of the kinetics of internal ice-formation.
- Species specific dehydration and hydration during freezing and thawing.
- Ultrastructural changes in sperm during freezing and thawing.
- Phase transitions in membrane lipids associated with the cold shock.
- Biochemical composition in the sperm of different finfish, molluscan and crustacean species.
- Permeability to water and cryoprotectants.
- Electromagnetic of microwave heating of aqueous cryoprotectant solution from cryogenic temperatures.
- Cryomicroscope analysis and differential scanning calorimeter for freeze-damage determinations.
- Biotechnology of sperm/embryo bank and the possible hazards.
- Potential and constrains of cryobiology for maintaining the genetic diversity in both captive and wild aquatic species.

EXTENSION EFFORTS

The cryopreservation of teleost sperms provides practical functions and also opens new research frontiers. Cryopreserved sperms could be very useful in hatcheries with application to broodstock management, selective breeding and gene conservation. Extension has been conducted through organizing seminars and workshops, holding training courses, publishing handouts and preparing TV programs. The followings should be put under considerations for further extension.

On the technical aspects

The appication of the cryopreserved sperm has been fully explored. Taking the example of the cryopreservation of sperms of salmonid fishes which was studied in 1960's and has therefore attracted increasing interests (Stoss, 1983; Baynes and Scott, 1987; Wheeler and Thorgaard, 1991). Although there are reports of successful cryopreservation in laboratory situation, there have been few examples of applicaions at the hatchery level (Anderson and MacNeil, 1984; Cloud *et al.*, 1990). In our studies on the black porgy and the grouper, private hatcheries begin to accept the

technology transfer of sperm cryopreservation although in a limited scale. But in the cases of other species, there is still a need to find out the balance between the significance and economics of the transfer of their respective technologies.

- Handling of broodstock to facilitate the collection of raw milt and ripen eggs with best quality and sufficient quantity.
- Trial to use large FE straws of bags for holding milt. The use of larger straws will decrease the time to freeze large quantities and reduce the task of thawing when fertilizing large hatches of eggs.
- Simplifying the freezing protocols and making it usable in the field without too expensive installation.
- Demostrating how to achieve a reasonable fertility under commercial hatchery conditions.

On strategy aspects

In the last decade, extension works have been done by organizing seminars, sponsoring workshops, offering training courses, giving out protocols, demonstrating in public communication programs and helping in setting-up facilities. It is generally believed that with extensive and deeper researches, the cryopreservation of teleost sperms may maximize the possible benefit. To achieve this significant aim, attension should be paid to the following strategies:

- Incentive: The need for readily available sperm of good quality should be recognized and emphasized.
- Application priority: Established technology should be applied in the field to a greater or lesser extent according to the species priority.
- Research and extension personnel: More manpowers should be involved to carry out a series of intensified basic researches and extension works for a sound practical application.
- Advisory committee: An advisory committee should be established to solicit inputs from various scholars, experts and well trained users.
- Sufficient funding: In the past two decades, the research groups on cryobiology received sufficient financial supports from TFRI, the Council of Agriculture and the National Science Council of the Republic of China. Research fundings should be continued to further explore the significant programs, elevate the technological standard and solve extension constraints.
- Exchange center: An exchange center of aquatic sperm bank should be established for domestic

exchange among scientists and farmers and international exchanges among countries, like the system implemented in the livestock field.

PROSPECTS

There are three stages for developing cryogenic preservation of fish sperms in Taiwan, namely collective, basic and active stages. A series of works have been done in the technology establishment, cooperation and extension; however, none at their fully active stage. This paper attempts to review the achievements of researches on the cryopreservation of fish sperms in Taiwan. It is hoped that this presentation will come up with some useful recommendations and distinguished advices so that further development will lead to directions shown below, which will in turn contribute to the aquaculture of Taiwan in the 21st century.

- Cryopreservation of fish sperm can be successfully used as a fishery management tool. Cryopreservation provides some means of control relative to mating and reproduction, including artificial propagations with individuals of same species from the distant locations or of different , mature seasons or developing a profitable new hybrid.
- Sperms from males in the wild can be cryopreserved for the optimal mature season of hatchery stocks so that genes from the fish in the field can be transferred into the hatchery population.
- Cryopreservation of fish sperms can be used to guarantee the specific fish stocks and to ensure against the loss of specific genes caused by natural, overfishing or pullution-causing diasters.
- Cryopreservation of fish sperms can be used in the hatchery management to solve the problem of having a disproportion between the males and the females.
- Cryopreservation of virus- or bacteria-free sperms can save valuable eggs.
- Induction of gynogenesis becomes highly feasible if the irradiated sperms is cryopreserved in advance.
- Functions of sperms and gene bank can be further facilitated.

Acknowledgements: The findings and conclusions of this review were made possible through the financial supports of the Taiwan Fisheries Research Institute, the

National Science Council, ROC and the Council of Agriculture, ROC. Thanks are due several assistants who worked with me on the following projects: NSC 71-0409-B056a-01. NSC 72-0409-B056a-02, NSC 73-0409-B056a-03, NSC 78-0409-B056-04, NSC 79-0409-B056-02, NSC 80-0409-B056-03, COA 77-7.1-Food-110 (G5), 78-7.1-Fish-06(5), 80-7.1-Food-121 (77). The valuable assistance of hatchery owners and TFRI colleagues are also gratefully acknowledged.

REFERENCES

Alderson, R. and A. J. MacNeil (1984) Preliminary investigation of cryopreservation of milt of Atlantic salmon (*Salmo salar*) and its application to commercial farming. *Aquaculture* **43**: 351-354.

Ashwood-Smith, M. J. (1980) *Low temperature preservation of cell, tissues and organs. In:* Low Temperature Preservation in Medicine and Biology. (M. J. Ashwood-Smith and J. Farrant eds.). Pitman Medical, Turbridge Wells, UK. pp. 19-43.

Baynes, S. M. and A. P. Scott (1987) Cryopreservation of rainbow trout spermatozoa: The influence of sperm quality, egg quality and extender composition on post-thaw fertility. *Aquaculture* **66**: 53-67.

Billard, R. (1984) *La conservation des gametes et l'insemination artificielle chez le bar et la daurade. In:* L'Aquaculture du Bar et des Sparides (G. Barnabe and R. Billard eds.). INRA Publication, Paris. pp. 95-116 (in French with English summary).

Chao, N. H. (1982) *New approaches for cryopreservation of sperm of grey mullet,* Mugil cephalus. *In:* Reproductive Physiology of Fish. (C. J. J. Richter and J. H. Th. Goos eds.). Wageningen, The Netherlands: Centre for Agricultural Publishing and Documentation. pp. 132-133.

Chao, N. H. and I. C. Liao (1987a) *Application of honey in cryopreservation of sperm of milkfish (Chanos chanos) and black porgy (Acanthopagrus schlegeli). In:* Proc. Rep. Physics of Fish. pp. 94-95.

Chao, N. H. and I. C. Liao (1987b) *Cryopreservation of marine and freshwater fish sperm. In:* Proc. Symp. Fish Repr. Endocrine Control: Basic Practical Aspects. (J. Y. L. Yu, C. M. Kuo, S. H. Shih and S. J. Chen eds.). June 19-20, 1987. Taipei, Taiwan, ROC. pp. 188-198 (*COA Fish. Ser.* **7**: 226-246) (in Chinese with English abstract).

Chao, N. H., H. P. Chen and I. C. Liao (1975) Study on cryogenic preservation of grey mullet sperm. *Aquaculture* **5**: 389-406.

Chao, N. H., H. P. Tsai and I. C. Liao (1992) Short- and long-term cryopreservation of sperm and sperm suspension of the grouper, *Epinephelus malabaricus* (Bloch and Schneider). *Asian Fish. Science* **5**(1) (in press).

Chao, N. H., W. C. Chao, K. C. Liu and I. C. Liao (1986) The biological properties of black porgy (*Acanthopagrus schlegeli*) sperm and its cryopreservation. *Proc. Natl. Sci. Council B: Life Sci.* **10**(2): 145-149.

Chao, N. H., W. C. Chao, K. C. Liu and I. C. Liao (1987) The properties of tilapia sperm and its cryopreservation. *J. Fish Biol.* **30**: 107-118.

Cloud, J. G., W. Miller and M. J. Levanduski (1990) Cryopreservation of sperm as a means to store salmonid

germplasm and to transfer genes from wild fish back into hatchery populations: A field demonstration. *Prog. Fish Cult.* **52**: 51-53.

Cognie, P. F., R. Billard and N. H. Chao (1989) La cryoconservation de la laitance de la carpe, *Cyprinus carpio*. *J. Appl. Ichthyol.* **5**: 165-176 (in French with English summary).

Erdahl, D. A. and E. F. Graham (1980) Preservation of gametes of freshwater fish. *Proc. Int. Congr. Anim. Reprod. Artif. Insem. RT-H* **2**: 317-326.

Guest, W. C., J. W. Avault Jr. and J. D. Roussel (1976) Preservation of channel catfish sperm. *Trans. Am. Fish. Soc.* **105**(3): 469-474.

Harvey, B., R. N. Kelley and M. J. Ashwood-Smith (1982) Cryopreservation of zebra fish spermatozoa using methanol. *Can. J. Zool.* **60**: 1867-1870.

Mires, D. (1974) On the high percent of tilapia males encountered in captive spawnings and the effect of temperature on this phenomenon. *Bamidgeh* **26**(1): 3-11.

Stoss, J. (1983) *Fish gamete preservation and spermatozoan physiology. In:* Fish Physiology, Vol. 9B (W. J. Hoar, D. J. Randall and E. M. Donaldson eds.). Academic Press, New York. pp. 305-350.

Wheeler, P. A. and G. H. Thorgaard (1991) Cryopreservation of rainbow trout semen in large straws. *Aquaculture* **93**: 95-100.