

Chilled and Cryogenic Storage of Gametes of Thai Carps and Catfishes

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CHILLED AND CRYOGENIC STORAGE OF GAMETES OF
THAI CARPS AND CATFISHES

by

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ABSTRACT

Withler, F. C. 1980. Chilled and cryogenic storage of gametes of Thai carps and catfishes. Can. Tech. Rep. Fish. Aquat. Sci. 948: 15 p.

Fertilizabilities of ova and sperm of Indian carp Labeo rohita, common carp Cyprinus carpio, Puntius Puntius gonionotus, grass carp Ctenopharyngodon idella, bighead carp Aristichthys nobilis and the catfish Pangasius sutchi were examined after storage in refrigerators or on ice or in liquid nitrogen (LN₂). The period of fertilizability of ova after extrusion was not extended significantly by chilled storage in a refrigerator. The period during which sperm could be activated after extrusion was extended to several hours by chilled storage in syringes. Carp sperm (Indian carp, common carp, Puntius, bighead carp) mixed with an extending solution containing DMSO could be activated after storage for various periods in LN₂. In one trial, Indian carp sperm stored in LN₂ fertilized ova as well as fresh sperm applied to a control group of ova. P. sutchi sperm in either of the two extenders used, and stored in LN₂, produced a gel which could not be used effectively in fertilizing ova. A description of current methods of inducing maturation by injection of macerated pituitary glands is given. Recommendations are made for further work on gamete storage applicable to Thai fish culture.

RÉSUMÉ

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D'après l'étude de la fertilité des ovules et du sperme de Labeo rohita, de Cyprinus carpio, de Puntius gonionotus, d'Aristichthys nobilis, de Ctenopharyngodon idella et de Pangasius sutchi, après réfrigération ou conservation sur la glace ou dans l'azote liquide (N₂), la réfrigération des ovules, après leur prélèvement par pression abdominale, n'en prolonge pas beaucoup la fertilité. Réfrigérer dans des seringues, le sperme peut rester fertile jusqu'à plusieurs heures. Le sperme des quatre premières espèces, gardé pendant diverses périodes de temps dans N₂ au moyen d'un diluant additionné de DMSO peut être réactivé: ainsi avec celui de L. rohita on a fertiliser des ovules avec le même succès qu'avec du sperme frais et des ovules témoins. Quant au sperme de P. sutchi, mélangé aux deux diluants utilisés et gardé dans N₂, il a formé un gel inutilisable. Les méthodes actuelles de déclenchement de la maturité par injection d'une macération de glandes pituitaires sont également décrites, puis suivent des recommandations de travaux à faire sur la conservation des gamètes dans le contexte de la pisciculture d'espèces de la Thaïlande.

INTRODUCTION

The writer was seconded to the Canadian International Development Agency (CIDA) for the months of July and August, 1976 as a short-term expert. The purpose of the secondment was to discover whether or not techniques for short- and long-term storage of sperm and ova could be applied in Thai fish culture practice. If preliminary work looked promising, it was intended that Thai fishery workers become familiar with the techniques so that they could carry on the experimental work and apply any positive results. Much of the experimental work was carried out during demonstration classes in which biologists from various fisheries stations participated. The writer then visited many of the stations to see first-hand how the results might be applied.

This report records the results of preliminary trials of storage of sperm and ova of some commercially important Thai freshwater fishes, and on the basis of the results suggests what further work may be needed to apply gamete storage techniques to fish culture practice. The report also contains comments on other aspects of tropical freshwater fish culture in the hope of assisting other workers from the Temperate Zone intending to carry out similar work--in a short term project, precious time can be lost becoming familiar with the very different life histories of tropical fishes and with the usual fish culture methodology. The report also records the names of those who attended the demonstration classes and participated in the experimental work.

BACKGROUND

The Freshwater Division of the Thai Department of Fisheries maintains a large number of fisheries stations whose main purposes are to provide young fish ("seed") to fish farmers and to plant fry in larger lakes and reservoirs. The farmers pay a nominal price for the seed for personal and commercial uses which range from seeding a single pond or rice paddy to meet the farmers' own need for fish, up to commercial fish farms of several hectares. In some cases (particularly in northeast Thailand) the seed is used in village cooperatives in which responsibility for rearing and harvesting the fish is vested in one or more individuals, while the profits derived are returned to the community for improvement of services.

Tropical fresh waters such as those of Thailand are enormously productive. Most of Thailand (excepting the north and northeast) is low and wet and farmed extensively, particularly for rice. The monsoon season when rainfall is heaviest extends from July through October, with air and water temperatures commonly about 30°C for long periods. Fresh waters become silt- and nutrient-laden from natural and agricultural runoff. In spite of the heavy rainfall, the skies are clouded for only 2-3 h a day, and provide abundant solar radiation to the slow-moving streams, shallow lakes and rice

fields. The waters teem with more than 560 species of fish (Smith 1945) and many invertebrates including freshwater shrimp and prawns. Growth of most fish is rapid, so that in some cases a planted fry may grow to 8 kg in 12 mo. Some species are harvested at 6 mo or less after planting. The productivity of the freshwater fisheries in addition to extensive marine fisheries places Thailand 10th in world fish production (Canada is 17th) and makes her largely self-sufficient in fish (FAO 1978). The Thai Department of Fisheries plans to expand fish production to the point where Thailand's exports of fish significantly outweigh her imports.

Recently the Department extended its freshwater fish culture operations to include non-native species, most notably the Chinese carps (Ctenopharyngodon idella (grass carp), Hypophthalmichthys molitrix (silver carp), Aristichthys nobilis (bighead carp) and the Indian carp Labeo rohita. The purpose of these introductions is to provide stocks which are reared easily in ponds yet which grow rapidly to a large size without preying on other harvestable fish. They also command a premium price in the markets. The Chinese carps have one disadvantage which the Department would like to minimize: they come from a temperate environment and do not mature naturally in Thailand's climate. To breed them it is necessary to induce maturation artificially. This is accomplished by removing the pituitary glands of other fish (sometimes the same species) to inject into potential breeders. The process is time-consuming and expensive because it requires holding stocks of fish which serve merely as pituitary donors. In addition the technique is still an art, not a science, and the precise time of full maturity is sometimes unpredictable.

In addition, some of the Department's stations are attempting to culture two large river-dwelling carps Catlocarpio siamenensis and Probarbus jullieni which occur naturally in the Chao Phraya and Mekong Rivers. If the work proves successful, fry of these carp (Catlocarpio may grow to 3 m in length and weigh 130 kg) will be planted in reservoirs behind existing and planned hydropower dams. Currently the main problem in culturing is to obtain enough mature male and female spawners at the same time to carry out breeding.

Native catfish such as Pangasius sutchi and Clarius spp. are cultured extensively, from both pond-held and wild stocks. Induced maturation is required for these species also, to ensure the large annual supply of seed required. Induction techniques are being demonstrated and used throughout most of the fisheries stations.

DEMONSTRATION CLASSES

The classes were held at the National Inland Fisheries Institute (NIFI) laboratory at Bangkhen, Bangkok. They were attended by members of the NIFI staff and biologists drawn from freshwater fisheries stations throughout Thailand. It was intended that, wherever possible, the writer would visit each station from which a biologist had been sent, so that the potential usefulness of gamete storage techniques could be assessed on site.

The classes combined lectures and experimental work. The lectures consisted of a review of current knowledge regarding gamete storage. "Short-term" storage of ova and sperm in refrigerators and on ice was discussed, with reference to use under field conditions. Cryogenic "long-term" storage techniques for sperm were reviewed, outlining the development of extenders and protective agents. Concurrently, the classes participated in tests of both techniques applied to fishes then available at the NIFI laboratory (3 spp. of carp and 1 catfish).

The first class was held from July 19-23, 1976. The participants, including 2 biologists from Bangladesh, were as follows:

	<u>Fisheries Station</u>
Mr. Manu Potaros	NIFI, Bangkok
Mr. Suchit Bhinyoying	NIFI, Bangkok
Mr. Jitt Petcharoen	NIFI, Bangkok
Mr. Samrouy Meenakarn	NIFI, Bangkok
Ms. Revadee Sriprasert	NIFI, Bangkok
Mr. Likit Nukulruk	Chainat
Mr. Paisan Juangpanich	Chainat
Mr. Wichian Plengchawe	Nakorn Sawan
Mr. Wirun Boonmahn	Nakorn Sawan
Mr. Rewat Ritaporn	Chaing Mai
Mr. Suebpong Chatmalai	Chaing Rai
Mr. Watana Leelapat	Karn Chantaburi
Mr. Islem	(Bangladesh)
Mr. Ahmed	(Bangladesh)

The second class was held from August 9-11, 1976. Two biologists from the Philippine Republic also attended. The participants were:

	<u>Fisheries Station</u>
Mr. Chanint Sritongsuk	Nong Khai
Mr. Tein-thong Yoo-Vetwatana	Nong Khai
Mr. Pichit Srimukda	Sakol Nakorn
Mr. Boonchuay Chou-Thavie	Udon Thani
Ms. Somsri Ngam-Vongchon	Udon Thani
Mr. Chamrus Khantichitt	Ubol Ratana
Mr. Manas Chantasut	Maha Sarakam
Mr. Vira Thoop-Buchar	Surin
Mr. Boonchuay Chao-Paknam	Khon Kaen
Mr. Pitaya Pen-Napaporn	Karat
Mr. Visant Meesawat	Tak
Mr. Panya Ussa-Warng-Goon	Rayong
Mr. Saming Songtawontawe	Rayong
Mrs. Wilaiwan Borom-Thanarat	Brackish Water Division
Mr. Somphong Suivan-Natote	Cha-Choengsao
Mr. Sujin Manevongsa	Songkhla
Ms. Pura Duldoco	(Philippines)
Mr. Romulo Samson	(Philippines)

Following each session, trips were made by the writer accompanied by one or more NIFI staff to fisheries station in various parts of the country. These were:

Chainat	July 26, August 26
Nakhon Sawan	July 27
Chaing Mai	July 29
Chaing Rai (Phayao)	July 30
Khon Kaen	August 4
Ubol Ratana	August 5
Udon Thani	August 5
Nong Khai	August 5
Songkhla (Brackish Water Div.)	August 20

MATERIALS AND METHODS

SOURCES OF GAMETES

Most of the gamete storage tests were carried out at the NIFI laboratory, so that milt and ova used in experiments were taken from brood stock held in the laboratory's ponds. In most cases it was necessary to induce maturity to obtain gametes.

To demonstrate the method of induction, the procedure employed to produce Indian carp gametes is given below as an example:

- (1) The required male and female breeders were caught by seine net in the holding ponds, sorted and transferred to a large fine-meshed net suspended in a concrete holding tank. Each fish was weighed during transfer to determine the dosage of crude pituitary gland required. Isolation of breeders some hours before spawning allows the fish to vent their faeces, so that gametes are not contaminated during spawn collection.
- (2) The required "donors" (fish providing the pituitary glands for injection) were assembled and weighed. The number (or weight) of donors required was determined from the weight of the breeders, on the basis of the number of "doses" required to bring the breeders to maturity. One dose is the pituitary gland of another fish equivalent in weight to that of the breeder, or the combined glands of several whose weight adds up to that of the breeder. Sufficient donors were assembled to provide a total of 2 doses per female breeder and 0.5 doses per male.
- (3) The first injection of female breeders was made 12 h before the ova were required. Sufficient glands were taken from recently killed donors to provide 0.5 doses per female. The glands were collected in a small glass homogenizer and macerated with a glass pestle. Enough 0.7N saline was added to make up the required volume--usually 1 mL per injection. (Sometimes mammalian chorionic gonadotropin is combined with crude pituitaries and would be added at this stage of preparation of the injection.)

The solution was then injected into the body wall musculature immediately behind the pectoral fin of the breeder. The treated breeders were returned to the holding net.

(4) The second injection of females was made 6 h after the first and consisted of 1.5 doses given in the same manner as the first. The first and only injection of males was made also at this time, and consisted of 0.5 doses per breeder.

(5) After the 6 h injections the breeders were watched almost continuously for signs of spawning. The ova are most fertilizable at the time the breeder begins to shed them voluntarily and the period for maximum fertilizability is short. Ova taken an hour earlier or later may be infertile.

(6) Ova were stripped by pressing the sides of the abdomen toward the vent and collecting the extruded ova in a dry Petri dish. Milt was stripped similarly, collected in beakers and, if not needed immediately for fertilizing, drawn into syringes for later use.

It should be noted that the crude pituitary injection method is unpredictable in effectiveness and timing. Success varies with the stage of maturity of breeders and donors, their age, the species of donors used, and the skill of the breeding manipulator. Probably the fishes' physical condition, water temperatures and amount of handling also affect the results. Experiments involving manipulation of gametes require detailed planning, careful preparation of breeders and equipment, and a flexible schedule.

CHILLED STORAGE

After being measured into required volumes (usually 25 or 50 mL) in a graduate, ova obtained from stripping were placed either in 250 mL glass beakers covered with aluminum foil or in plastic (Whirlpack) bags. They were then placed in a household-type refrigerator for storage until required. Milt was drawn into 1-mL disposable plastic syringes and placed in the refrigerator.

A maximum-minimum thermometer kept in the refrigerator recorded temperature ranges from 2-9°C. The refrigerator was used to store other materials and was opened frequently--during the course of the tests the average outside air temperature was about 30°C, ranging from 27-33°C.

When milt which had been stored under refrigeration was to be tested for fertilizability, a small portion was applied to ova which had been collected in a small beaker. The milt and ova were mixed by stirring with a feather, tap or pond water being added soon after the milt was applied. After a short period of gentle stirring of the ova and milt in water, the ova were poured into a hatching funnel for incubation.

CRYOGENIC STORAGE

Milt was drawn into a 10-mL syringe up to the required amount then discharged into a dry beaker. The "diluent" (see below) was measured similarly by syringe and discharged into the beaker containing milt (usually 4 parts diluent: 1 part milt). The solution was mixed thoroughly then drawn into a syringe and discharged into 1-mL glass ampoules commonly used to preserve bull semen. The unsealed ampoules were placed in supporting "canes" and suspended in the vapour 2 cm or more above the liquid nitrogen (LN₂) in the cryogenic container until frozen (5-10 min). The loaded canes were then submerged in the LN₂ until required.

The LN₂ container was a Linde model LD-30, maximum capacity 31.8 L. The LN₂ was maintained by filling the container at a commercial factory in Tonburi across the Chao Phraya from Bangkok. The LN₂ cost 40 Thai baht (about \$2.00 U.S.) per L; specified working evaporation rate for the LD-30 is 0.63 L/day.

The diluent consisted of an "extender" and a "protective agent", usually in the ratio 9 parts extender: 1 part protective agent. (An extender could be defined as a solution of inorganic salts and organic substances, more or less isotonic to the seminal fluid; a protective agent is a substance which penetrates the sperm cells and minimizes freeze-thaw damage.) Most often the protective agent used was dimethyl sulfoxide (DMSO); glycerol was used rarely. In this report, unless glycerol has been identified specifically as the protective agent, it should be assumed that DMSO was used in preparation of diluents.

The extenders were made up by adding 100 mL of distilled water to batches of chemicals which had been weighed, combined and packaged beforehand. Only the two extenders¹ which had been most effective in cryopreservation of salmon sperm in tests at the Pacific Biological Station were tried: 189M and 251. The compositions of the dry batches of extenders were as follows:

	<u>Extender 251</u>	<u>Extender 189M</u>
	mg	mg
NaCl	850	730
NaHCO ₃	500	500
Fructose	-	500
Vegetable lecithin	1500	750
Mannitol	-	500

Thawing the frozen sperm for use involved removing the ampoules from the LN₂, grasping them at the open end and swirling them in tap or pond water until the contents were partially thawed. The neck of the ampoule was then broken off and the contents poured directly onto recently stripped ova and mixed into them with a large feather. Water was then added to the mixture of ova and milt, which was gently stirred. The ova were then poured into a hatching funnel for incubation.

¹See Ott, 1975. Extender 189M used here was a special formulation recommended earlier for Pacific salmon milt by Dr. H. F. Horton, Dr. Ott's supervising professor and colleague in sperm cryopreservation studies.

SPERM ACTIVITY

Samples of sperm were examined by microscope at 400x. Drops of milt were obtained from a syringe needle (chilled milt was stored in plastic syringes) or from a capillary tube dipped into an ampoule used to store LN₂-frozen milt. A drop of milt was placed on a glass slide and covered with a cover glass. The milt was examined undiluted or after dilution by flooding under the cover slip with tap water, or distilled water. Activity was measured by estimating subjectively the proportion of sperms displaying movement, whether vigorous or sluggish.

INCUBATION OF TEST OVA

Test ova were placed in hatching funnels suspended in roofed concrete holding ponds. The hatching funnels consisted of fine-meshed cloth hung on wire frames, 40-60 cm in diameter and about 70 cm deep. A small brass funnel suspended at the bottom of each net and attached to plastic tubing directed a flow of water upward into the net. The flow was adjusted to cause the ova to be suspended in the water column, free of the nets' sides and bottom. Adhesive ova would attach to the nets' sides where they would be bathed in a slow current.

To assess fertilization rates, samples of ova or hatched larvae would be withdrawn at intervals and examined under a low power microscope.

RESULTS

Observations were made on gametes of several species whose scientific, English and Thai names are given below:

<u>Labeo rohita</u>	Indian carp	Rohu or Yee-sok tet
<u>Pangasius sutchi</u>	Pangasius	Pla sawai
<u>Cyprinus carpio</u>	Common carp	Pla nai
<u>Puntius gonionotus</u>	Puntius	Pla tapien khao
<u>Ctenopharyngodon idella</u>	Grass carp	Pla chao-hue
<u>Aristichthys nobilis</u>	Bighead carp	Pla soong-hue

SHORT TERM STORAGE OF OVA

All attempts to store ova for significant periods by refrigeration were unsuccessful. Indian carp ova kept in a beaker under refrigeration were as fertilizable after 35 min as fresh ova; ova from the same batch

stored for 1 day were unfertilizable. Pangasius ova stored overnight in the refrigerator were unfertilizable the next day. Common carp ova refrigerated for 120, 130 and 230 min were unfertilizable.

SHORT TERM STORAGE OF MILT

In a single test, Indian carp milt was fully effective in fertilizing ova after 23 min of refrigeration (92% fertilization of 50 mL of test ova). No further tests were made.

Pangasius milt, stored in syringes, showed sperm activities after storage in a refrigerator as follows: 10 min, 100%; 50 min, 100%; 80 min, 100%; 18 h, 10%; 3.5 days, 0%.

Common carp milt, held in a syringe in a mixture of crushed ice and water, gave readings of sperm activity as follows: 0 min, 100%; 15 min, 100%; 53 min, 80%; 155 min, 90%; 4 h (ice now melted), some activity. In another test common carp milt held in syringes in a refrigerator showed estimated sperm activities as follows: 0 min, 100%; 100 min, 100%; 210 min, 100%. In another refrigeration test, activities were as follows: 0 min, 100%; 75 min, 100%; 24 h, 2%.

Puntius milt held in a syringe in a refrigerator showed the following activities: 0 min, 100%; 5 1/2 h, 15%.

LN₂ STORAGE OF MILT

Indian carp. Observations were made of sperm activities following LN₂ storage of milt in diluents containing extenders 251 and 189M. In one test, milt mixed with 189M diluent, frozen in LN₂ and examined 1 day later showed a sperm activity of 20%. In another, milt mixed with 251 diluent showed an activity of 0% after being frozen for 1 h, whereas milt mixed with 189M diluent showed activities of 10-20%. In a third test, milt frozen in 251 diluent showed 1-2% activity after 26 days of storage while milt in 189M diluent taken at the same time and frozen for the same period showed activities of 7 1/2%, 10% and 7 1/2%.

Milt stored in LN₂ was tested for its ability to fertilize fresh ova. The milt was collected, mixed with 189M diluent and stored overnight. Ova were stripped from a female and a 50 mL portion was treated with 1.5 mL of thawed milt. To serve as a control, a smaller portion of ova was treated with fresh milt. The two ova lots were washed, then placed in two incubation funnels. Samples of ova from the 2 nets were examined at intervals. The results were as follows:

Time (h)	LN ₂ treated			Control		
	Live	Dead	% Live	Live	Dead	% Live
3 1/2	205	54	79	247	107	70
5 1/4	35	32	52	37	69	35
8 1/4	80	51	61	91	130	40
8 1/4	64	47	58	75	87	46

Normal larvae emerged from the live ova from both control and LN₂-treated groups.

In a later test carried out in the same way, but involving 25l diluent as well as 189M diluent, all ova died including the controls. It was assumed that the fresh ova used in the test were infertile.

Pangasius. In one test milt mixed with 25l diluent and frozen overnight showed the following activities: 19 h, 1%; 23 h, less than 1%. In another, milt mixed with 25l diluent, frozen and examined 4 h later showed an activity of less than 1%.

Milt lots mixed with 25l and 189M diluents, frozen for 1 and 2 days respectively, were applied to 50 mL lots of fresh ova. At the same time, a 50 mL lot was treated with an equivalent amount of fresh milt. The 3 lots of ova were placed in separate incubation funnels. Four h later (at the morula stage) samples from the 2 treated and 1 control groups indicated that the ova were nearly all alive. However 22 h after the fertilizations had been made, only about 1% of the ova treated with LN₂-stored milt were still alive (as larvae) whereas most of the control ova had hatched into healthy larvae.

It was noted that in most ampoules of LN₂-stored Pangasius milt, the contents on thawing had turned into a soft jelly-like mass in which there were only about 1% active sperm. The jellied contents could not be mixed thoroughly into a mass of ova. To examine the possibility that the concentrations of protective agent (DMSO) affected consistency, or that another protective agent (glycerol) might not have the same effect, a series of 189M and 25l diluents containing different concentrations (1%, 5%, 10%) of either DMSO or glycerol were prepared. Milt was mixed with these diluents in the standard manner, frozen in LN₂, then examined within 25 h. In all cases the thawed material assumed a jelly-like consistency.

Common carp. In one test, milt was mixed with 25l diluent, frozen and later examined for sperm activity: 1 h, 10%; 24 h, (4 samples) 5%, 20%, 1%, 10%. In another using 189M diluent, the activity after 1 1/2 h was 20%.

Puntius. In one observation of sperm activity after LN₂ freezing, Puntius milt was mixed with 189M diluent and frozen in LN₂. One hour after preparation and freezing, sperm activity was about 20%.

Milt mixed with 189M and 25l diluents was stored overnight in LN₂. One ampoule of each was applied to two 5 mL lots of fresh ova, while 0.2 mL

of fresh milt was applied to a third lot of ova to serve as a control. The 3 ova lots were placed in incubation funnels. A replicate of the above test was prepared and the resulting lots of ova placed in another 3 funnels.

In the first test, nearly all ova whether treated or control, were dead after 4 1/2 h. In the replicate, most of the control ova (about 95%) were still alive and normal in appearance. The ova treated with milt which had been stored in LN₂ were nearly all dead (about 95%). It was assumed that the ova used in the first test were infertile.

Grass carp. Less than 2 mL of milt was obtained from a grass carp at Chainat fisheries station. One-half was mixed with 189M diluent, the other with 251 diluent; the ampoules were frozen in LN₂ in the normal way. Microscopic examination 21 days later showed no sperm activity in either sample.

Bighead carp. A small amount of milt was collected at Chainat fisheries station, mixed with either 251 or 189M diluent (which is unknown), and stored in LN₂. Examination 21 days later showed that about 1% of the sperm were active.

CONCLUSIONS AND RECOMMENDATIONS

SHORT-TERM STORAGE OF OVA

All attempts to store ova for significant periods were failures. This was at least partly due to the fact (previously unknown to the author) that in tropical carps and catfishes, development of mature ova begins soon after extrusion, fertilized or not. Once cell division has begun, of course, fertilization cannot occur. Parthenogenetic development proceeds to the morula or gastrula stage, after which the ova break down.

To store unfertilized ova successfully it will be necessary to inhibit cell division, at least. Several techniques might be tried:

- (1) Chilling the ova immediately after extrusion. In our abortive attempts, we simply placed 50 mL of fresh ova in a covered beaker and refrigerated them. The thickness of the layer was such that immediate chilling didn't occur. Perhaps if the ova layer were kept very thin, so that it cooled quickly, cell division could be inhibited. Placing the ova in a plastic bag in a layer only 2-3 mm thick and refrigerating them immediately could be tried.

- (2) Keeping out all extra moisture. In our trials there was a large air space in the beaker above the ova. Moisture condensed on the inside of the beaker after it had cooled in the refrigerator. It's possible that this moisture was sufficient to permit the ova to swell somewhat and allow cell division to occur. Further trials should include attempts to avoid condensation by excluding air from the container.

(3) Placing the ova in non-toxic isotonic or hypertonic solutions. It is possible that the moisture content of the ova could be held at the level existing at the time of extrusion by placing them in solutions with concentrations which would prevent water from entering the ova, or even withdraw some by osmosis, and hence prevent cell division. Swelling would occur after the sperm and water were applied at fertilization.

The above suggestions are speculative and should not have a high priority until the more likely prospect of sperm preservation have been explored.

SHORT-TERM STORAGE OF MILT

Our observations showed that short-term storage of carp and catfish milt in a refrigerator or on ice is feasible. We found that sperm could be activated for up to 24 h after extrusion, if chilled. Collected milt should be kept free of contaminants such as blood, mucus, faeces or moisture--addition of water, for example, brings forth furious activity which soon uses up the sperms' energy and they become inactive in less than a minute, chilled or not.

Further work should be done to define the optimum temperature for chilled storage. Perhaps a range of temperatures from 1-10°C at 2°C intervals could be tested for each species. Once the best temperatures had been found, it would be desirable to design ice containers which would provide the desired temperatures in milt stored in them.

Thai biologists have done some work in developing solutions which tend to keep the sperm alive after extrusion. Presumably the best solutions would be those which were non-toxic and kept the sperm alive but inactive until water was added. These solutions used together with chilling might extend the useful storage period.

A high priority should be placed on developing techniques for short-term storage in refrigerators or on ice, and on 'extending' solutions. Refrigerators and ice are quite readily available at the fisheries stations, so that putting the techniques into practice would be practicable.

LONG-TERM STORAGE OF MILT (LN₂ FREEZING)

In the course of our experimenting we found that the milt of carps and that of the catfish *Pangasius* responded very differently to LN₂ freezing. Consequently the two groups will be dealt with separately.

Carps. We found that milt stored in the two extenders developed for Pacific salmon--189M and 251--showed sufficient activity (5-20%) after LN₂ storage to suggest that long-term storage of carp sperm was

feasible. The species showing potential were Indian carp, common carp and Puntius. Storage of bighead carp milt may also be possible under better conditions. In a test where LN₂-preserved Indian carp milt was used to fertilize fresh ova, 58% of the ova ultimately produced healthy larvae, comparable to the hatching rate of a control group. Several variables should be examined to optimize the technique for carps:

(1) Extenders. The two extenders tested, out of the many which could be concocted, were those which worked well for Pacific salmon milt. Undoubtedly others which would be better for carps could be developed. It may be that an optimum extender would be different for each species--our tests indicated that 189M was better for Indian carp milt than was 251. Horton, Grayhill and Wu (1967) outline many that they tested for salmonids, some of which could be tried. Perhaps of more importance however, is their method of attacking the problem: find an isotonic solution which maintains the sperm alive but inactive, buffer it to the degree necessary to counteract the acidity or alkalinity of the protective agent (DMSO makes the solutions acidic), add the appropriate protective agent, then test the activity and fertilizing ability of sperm frozen in the test solution against the effectiveness of fresh sperm used on the same fresh ova.

Because both 251 and 189M appear to be at least partially effective for carps, they should be modified slightly to try to make them more effective, before attempting to develop entirely new extenders. Development of new extenders should be given a lower priority than testing other variables noted below.

(2) Protective agents. Most of our tests involved DMSO, although some glycerol tests were made. The results with glycerol were inconclusive. For carp sperm, on the basis of present data, DMSO appears to have the greatest potential. It is therefore recommended that tests using different diluent concentrations of DMSO ranging from 2-12% at intervals of 2% be given a high priority. These tests probably should be carried out for each carp species.

(3) Rates of freezing and thawing. Other workers have found that rates of freezing and thawing the diluted sperm were important in providing active sperm. Our tests with Pacific salmon milt indicated that these variables were not crucial, within limits, except that it was better to freeze the milt-diluent mixture soon after milt collection and to thaw the frozen mixture quickly.

For creating different rates of freezing, the simplest (but crudest) method is to control the distance the ampoules are held above the LN₂ during the initial freezing process. This is done by placing the ampoules in the canes at different levels, from 2 cm above the liquid up to perhaps 20 cm above, probably right up into the neck of the tank. Usually at least 3 ampoules can be placed in one cane--the lowest can be tested against the middle one and the top one. The closer the ampoule is to the LN₂ surface the faster the contents freeze.

Rates of thawing can be tested by thawing the ampoules in water baths at different temperatures. For salmon, we studied thawing-water temperatures from 10-45°C. We found that 45°C was best in providing sperm capable of fertilizing ova, although the differences were not great. It was important, however, to put the thawed sperm on the ova immediately, because it appears to live only a few seconds after thawing.

(4) Volumes of frozen milt. How well different volumes of sperm freeze should be examined. For the man in the field, larger units than 1-2 mL would be better since it would reduce the number of individual fertilizations necessary. Reducing the number of fertilizations needed for a given batch of ova would save time, which is important for carp eggs which begin development so soon after extrusion.

(5) Species. In the demonstration courses at NIFI we used species that were readily available from the brood stock ponds (common carp, Indian carp and *Puntius*). For these species sperm storage is not so important because they can be induced to spawn by themselves (common carp, *Puntius*) or brought to maturity readily by pituitary injection (Indian carp). The Chinese carps are more difficult to spawn, particularly the grass carp. It is important that the milt of these latter species be subjected to the LN₂ technique as soon as possible.

There are two carps from which spawn is taken mostly from wild stock: Catlocarpio siamenensis from the Chao Phrya River and Probarbus jullieni from the Mekong. Both grow very large and specimens must be obtained alive from fishermen when available and at widely separated fishing sites. Getting ripe males and females together for egg-taking is very difficult or impossible. If the milt could be stored in LN₂ until a ripe female were obtained, much of the problem of culture would be overcome.

A high priority should be placed on attempting to store the sperm of Catlocarpio and Probarbus.

Catfish. While the LN₂ storage techniques tried during this investigation look promising for carps, they appear to have been singularly unsuccessful when applied to *Pangasius*, the only catfish studied. Whether or not the poor response of P. sutchi milt applies generally to the Thai catfish groups is unknown.

We have not observed the jelly-like condition in extended, frozen milt in any of the Pacific salmon species studied earlier (pink, sockeye, chum, coho) or amongst the carps observed at NIFI. It is possible that the pH of the diluents 189M and 251 is such as to convert the sperm protein into a gel. Since both DMSO and glycerol were tested at concentrations ranging from 2-10% without noticeable effect on gel formation, it is unlikely that the reaction was caused by the protective agents alone.

Radically different extenders may have to be developed. These should be solutions in which the sperm remain alive but inactive--they will probably be isotonic to catfish milt and of the same pH. Once the desired

tonicity and pH have been achieved, the solutions should be adjusted to contain either DMSO or glycerol as protective agents. Protective agent concentrations of 2-10% should be tried. Once a solution that favoured some sperm activity after freezing has been developed, testing of variables such as those outlined above under 'Carps' should be carried out to achieve maximum activity and fertilizing ability.

Development of an effective sperm storage technique for catfish should have a high priority. Clarius and Pangasius spp. are very important in Thai fish culture and sometimes breeding, particularly for Clarius, is difficult. Preservation of Clarius milt in extenders 251 and 189M with DMSO and glycerol as protective agents should be tried (the reaction of Pangasius milt may have been anomalous). If the above trials are unsuccessful, development of suitable techniques for long-term preservation of catfish milt can be expected to be long and tedious.

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