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Short Communication

Sort term cryo-milt preservation of *Pangasianodon hypophthalmus* at the freshwater station of BFRI in Bangladesh

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Abstract: A total of 50 sub-adults of *Pangasianodon hypophthalmus* averaging 3.0 kg were stocked in a pond having an area of 0.2 ha. During winter months, fishes were fed with commercially available pelleted feed containing about 28% protein at the rate of 1.5% body weight daily while feeding rate was 3% during summer months. For augmentation of gonadal maturation, water shower was provided in the pond during winter months. Females were found with bulging belly and male with running milt during spawning season. Milt sample was expelled from mature and healthy brood by gentle abdominal pressure and collected into a clean and dry tube of 5 ml capacity. The milt samples were primarily placed in sealed ice box for 2-3 minutes then it was stored in a normal freezer for 20 minutes at 0 to 4^0C and then milt was stored in a deep freezer at -15^0C. Dimethyl-sulphoxide (DMSO) was used as cryo-protectant and phosphate buffered saline was used as extender. Cryo-tubes were leveled as A, B and C for storing 4, 8 and 12 hours, respectively. Leveled cry-tubes ware placed in the refrigerator for 15 min to reduce temperature to 4°C before fast freezing in the freezer. Then the tubes were stored in a deep freezer at -15°C. Sperm motility of fresh milt was recorded as 95%. In the thawing process, these cryo tubes were first transferred into refrigerator for 20 min to allow the milt to thaw from -20°C to 4°C inside the refrigerator. Thereafter, cryo tubes were transferred at room temperature and allowed to stand 5 min before mixing with female eggs. Thawed milt was added to the fresh eggs of *Pangasius hypophthalmus* and thoroughly mixed. After approximately 3 minutes, water was added to water-harden the eggs. Motility of cryo-preserved milt was nil. It is probably due to improper method applied to preserve the milt. For short-term preservation, milt was stored in ice box for 2, 4 and 8 hours. Motility rate of *Pangasius hypophthalmus* ranged between 5 and 50% and fertilisation rate varied between 1 and 40%, respectively.

Keywords: cryo-milt; preservation; *Pangasianodon hypophthalmus*; fertilisation rate

1. Introduction

The rapidly expanding aquaculture industry, particularly in the tropics, is increasingly relying on seed production from farmed fish stocks. There is growing evidence that many of these cultured fish stocks are now significantly degraded because of poor brood stock management practices. In the future it may be impossible to replace or invigorate these stocks as wild stocks are likely to be lost through either over exploitation or the destruction of their natural habitats. The maintenance of fish stocks in a genetically stable form (cryo-preserved) will therefore be imperative to secure the sustained long term development of fish production. Cryopreservation technology is now available and can be used to develop field based technologies to establish frozen sperm banks for commercially important tropical finfish species. In addition to preserving the present fish stocks, milt representing genetically improved fish stocks or selected traits can be cryo-preserved and stored with reduced chances of accidental loss either through genetic contamination or other unforeseen disasters such as the bio-diversity of aquatic organisms and such technology will be essential for the conservation of threatened aquatic

organisms. A fundamental requirement for effective gene-banking will be the reliability, accessibility and simplicity of the techniques for the cryopreservation of fish milt. Fish gene banks offer vast potential benefits to hatcheries (Chao and Liao, 2001). It offers genetic variability to fish hatcheries around the world. The use of frozen semen in breeding programmes offers a means to further broaden the genetic base of the targeted species. Genetic improvement of broodstock or hatchery species for traits such as disease resistant, fast growth rate, salinity tolerance etc. could also make feasible with the establishment of the cryogenic sperm bank. The applications of sperm cryopreservation in aquaculture were also highlighted by (Mongkonpunya *et al*., 2000). In the case of some species, males and females reach maturity over different periods of time; the cryo preserved semen could facilitate artificial fertilization and seed production (Tiersch, 2000). Cryo preserved sperm also provides flexibility in breeding programmes (FAO, 1971). It has been estimated that spermatozoa can last from 200 to 32,000 years (Stoss and Donaldson, 1983; Suquet *et al*., 2000). Low temperature approaches have been successful in fish sperm cryopreservation as the technology offers the best means for long term storage of fish semen. Frozen sperm to hybridize spring and fall spawning herring has been used by Blaxter (1953). It has been applied for more than 200 fish species (Figiel and Tiersch 1997, Chao and Liao 2001). Fish sperm cryopreservation has been investigated mainly in salmonids (Cabrita *et al*., 1998, Drokin *et al*., 1998) and some other fresh-water fish species (Aoki *et al*., 1997, Chao *et al*., 1987). The present study is to investigate the sort-term cryo-milt preservation of *Pangasianodon hypophthalmus* at the freshwater station of BFRI in Bangladesh.

2. Materials and Methods

2.1. Brood rearing

A pond of 0.2 ha area of the freshwater station of BFRI (Bangladesh Fisheries Research Institute) has been prepared by repairing dykes in December 2014. Every year brood pond is damaged due to the activity of rate and fresh water eel. Therefore in dry season brood pond need repair. The pond was disinfected by applying lime at the rate of 247kg/ha. The pond was filled up with water from a deep tube well. Water level was maintained at 1.8 to 2.0 m. A total of 50 broods of *Pangasianodon hypophthalmus* (sutchi) averaging 4.0 kg have been reared in the pond. Among the 50 P. hypophthalmus brood, 35 were female and 15 were male. Fishes were fed with commercially available pelleted feed containing about 28% protein at the rate of 2% body weight daily. Weekly water showering was provided in the pond for proper gonadal development of fishes. Moreover, water shower increases concentration of dissolve oxygen in pond water. Monthly sampling was done to check gonadal development of fishes. Female *P. hypophthalmus* were checked by observing external features of abdomen and, color and shape of genital papillae while their male counterparts were checked by gentle pressing the abdomen to get milt.

2.2. Collection of milt and motility examination and preservation

Mature and healthy male brood fishes were selected to collect milt. Milt sample were expelled from the male fish by gentle abdominal pressure and collected into a clean and dry tube of 5 ml capacity. Contamination of sample with blood, water, urine or the feces were avoided as these contaminants significantly reduce the milt quality and cause poor post-thaw sperm motility. The milt samples were primarily placed in sealed ice box for 2-3 minutes then it was stored in a normal freezer for 20 minutes at 0 to 4°C and then milt was stored in a deep freezer at -20°C. Before storage, cryo-diluent was prepared. Dimethyl-sulphoxide (DMSO) was used as cryo-protectant and phosphate buffered saline was used as extender. Cryo-diluents solution was prepared by mixing 75% phosphate buffered saline, 10% DMSO and 15% skimmed milk. This composition was homogenously mixture by vortex mixture. Milt was stored in 5 ml cryo-tubes. Cryo-tubes were leveled as A, B and C for storing 4, 8 and 12 hours, respectively. The milt was mixed with the solution at the ratio 1:1(v/v); and stored in 5 ml leveled cry-tubes. This cry tubes ware gently shaken to allow milt to properly mix with solution, and then carry those cryo-tubes by ice box. Leveled cry-tubes ware placed in the refrigerator for 15 min to reduce their temperature to 4°C before fast freezing in the freezer. Then this tubes ware storage in the -20°C deep freezer.

2.3. Thawing and fertilization

Samples of milt were removed from the deep freezer. In the thawing process, this cryo tubes were first transferred into refrigerator for 20 min to allow the milt to thaw from -20°C to 4°C inside the refrigerator. Thereafter, they were transferred at room temperature and allowed to stand 5 min before mixing with female eggs.

3. Results and Discussion

Healthy male of *Pangasianodon hypophthalmus* and were selected during peak breeding season. Male breeders were hypophysed with cPGE at the rate of 2 mg per kg of body weight to get milt easily. Milt was collected after 6 hrs of hypophysation. Mill was collected from the male by stripping into several ice cold sterilized tubes. Separate tubes were used for *P. hypophthalmus*. Sterilized tubes containing milt were stored in ice box for 2, 4 and 8 hours. Motility of Spermatozoa was assessed by a microscope. Motility rate of *P. hypophthalmus* ranged between 5 and 50% and fertilization rate varied between 1 and 40%, respectively. Hatching rate were very poor that ranged between 0 and 20% (Table 1).

Table 1. Details of experiment with preserved milt and fresh eggs of *P. hypophthalmus*.

Expt. No	Fresh eggs	Preserved milt (Hours)	Motility (%)	Fertilisation (%)	Hatching rate (%)
1	*P. hypophthalmus*	2	50	40	20
2	*P. hypophthalmus*	4	30	20	5
3	*P. hypophthalmus*	8	5	1	0

The cryoprotectants, DMSO and methanol at 5 and 10% concentrations produced better motility of sperm of rohu during 5 and 10 min incubation but 15% concentration seemed toxic and yielded poor motility which is similar to the findings of Yang *et al*.,(2007) and Sarder *et al*.,(2012, 2013) . Leung (1987) observed the best post-thaw motility of barramundi sperm at 5% DMSO while 10% cryoprotectant was effectively used by Withler and Lim (1982) Sperm of Olive barb (*Puntius sarana*) incubated with 5% DMSO and methanol produced longer motility but an acute toxicity was observed at 15% concentration. Cryoprotectants (DMSO, methanol, ethanol) at 5 and 10% concentration performed better during cryogenic freezing of *N. nandus* and *O. pabda* spermatozoa.

An accepted performance of Alsever's solution in cryopreservation of Indian major carp sperm was reported by Kumar (1988). Similarly, Alvarez *et al*., (2003) obtained good results from Alsever's solution with 10% DMSO during preservation of silver carp (*Hypophthalmichthys molitrix*) sperm. Along with Alsever's solution DMSO produced highest post-thaw motility in the present study which is considered as a common and effective cryoprotectant for cryopreservation of fish sperm and cell lines. Rao (1989) suggested that along with Alsever's solution DMSO might have positive impact on preservation as it penetrates rapidly into the cellular membrane and brings a quick balance between the intra and extra-cellular fluid concentrations. Though result was not satisfactory but farther research should conduct for prepare suitable protocol of *Pangasius hypophthalmus* milt preservation.

4. Conclusions

Results of the experiments with short-term preserved milt and fresh eggs of *P. hypophthalmus* were not satisfactory for commercial operation of fish hatcheries. However, it is a preliminary result and needs modification of short-term preservation method. Therefore, more experiments are to be conducted for getting scientifically explainable results on short-term preservation of fish milt in Bangladesh condition.

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Conflict of interest

None to declare.

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