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Sperm cryopreservation of the critically endangered olive barb (Sarpunti) *Puntius sarana* (Hamilton, 1822) ☆

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ABSTRACT

The present study focused on development of a sperm cryopreservation protocol for the critically endangered olive barb *Puntius sarana* (Hamilton, 1822) collected from two stocks within Bangladesh and reared in the Fisheries Field Laboratory, Bangladesh Agricultural University (BAU). The sperm were collected in Alsever's solution prepared at 296 mOsmol kg^{-1}. Sperm were activated with distilled water (24 mOsmol kg^{-1}) to characterize motility. Maximum motility (90%) was observed within 15 s after activation, and sperm remained motile for 35 s. Sperm activation was evaluated in different osmolalities and motility was completely inhibited when osmolality of the extender was $\geqslant$287 mOsmol kg^{-1}. To evaluate cryoprotectant toxicity, sperm were equilibrated with 5%, 10% and 15% each of dimethyl sulfoxide (DMSO) and methanol. Sperm motility was noticeably reduced within 10 min, when sperm were equilibrated with 15% DMSO, indicating acute toxicity to spermatozoa and therefore this concentration was excluded in further trials. Sperm were cryopreserved using DMSO at concentrations of 5% and 10% and methanol at 5%, 10% and 15%. The one-step freezing protocol (from 5 °C to −80 °C at 10 °C/min) was carried out in a computer-controlled freezer (FREEZE CONTROL® CL-3300; Australia) and 0.25-ml straws containing spermatozoa were stored in liquid nitrogen for 7–15 days at −196 °C. The highest motility in thawed sperm 61 ± 8% (mean ± SD) was obtained with 10% DMSO. The fertilization and hatching rates were 70% and 37% for cryopreserved sperm, and 72% and 62% for fresh sperm. The protocol reported here can be useful for hatchery-scale production of olive barb. The use of cryopreserved sperm can facilitate hatchery operations, and can provide for long-term conservation of genetic resources to contribute in the recovery of critically endangered fish such as the olive barb.

Introduction

Olive barb, *Puntius sarana* (Hamilton, 1822), locally known as Sarpunti, is a popular food fish and the largest barb native to the Indian sub-continent. The olive barb is a widely distributed cyprinid in the inland waters of South-East Asia [24]. The fish attain a maximum length of 42 cm and weight of 1.4 kg [37], reach maturity within a year, normally breed from April to mid-September [41], and fecundity ranges between 16,000 and 290,000 eggs per female [11]. Until the mid-1990s, farmers continued to include olive barb in carp-polyculture ponds. Currently, overexploitation, habitat degradation and various ecological changes have greatly reduced its natural populations [10], and as a result the fish has been enlisted as vulnerable in India [36] and critically endangered in Bangladesh [6].

Cryopreservation of sperm is a useful technique to preserve genetic material of endangered fish species [47] and can be utilized for fertilization [49] in well-designed breeding programs. During protocol development for fish sperm cryopreservation, key parameters of sperm samples (e.g., ionic composition, osmolality) [3] as well as development of appropriate activation media, immobilization solutions, cryoprotective agents, equilibration time, cooling rates, and thawing rates [44] should be given consideration because of differences within and among species. The most widely used cryoprotectant, dimethyl sulfoxide (DMSO), permeates the cells quickly and is usually used at concentrations 5–12%. It has been found to be effective for common carp (*Cyprinus carpio*) [23], spotted sea trout (*Cynoscion nebulosus*) [51], Atlantic salmon (*Salmo salar*) [17] and other fish species. Methanol is also a

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permeating cryoprotectant, known for low toxicity and has been reported to be used successfully at concentrations of 5–20% for the cryopreservation of cyprinid species [23], zebrafish (*Danio rerio*) [21], salmonids [28], catfishes [45] and tilapias [20]. Usually, after equilibration in cyroprotectant, the diluted sperm is frozen in pellets on dry ice [5], in straws with different cooling rates (5–15 °C/min), or in the vapor of liquid nitrogen [9,26] and stored. Later, the samples are thawed at 4 °C [38], 5 °C [52], 15–25 °C [27], 25 °C [9] or 37 °C [42] and used for fertilization of eggs. Sperm cryopreservation protocols are now available for over 200 species of finfish and shellfish [44] around the world. In Bangladesh, research on fish sperm cryopreservation was started in early 2004 and protocols are available for some of the commercially reared species. Unfortunately previous studies on sperm cryopreservation have not been carried out with imperiled fish species in Bangladesh with the vision to conserve their germplasm and genetic resources. Cryopreservation research in Bangladesh has concentrated on selection of suitable combinations of extenders and cryoprotectants, optimal dilution ratios of milt, and optimal cryoprotectant concentrations. Moreover, breeding success has not been adequately addressed in those studies [25].

Similar to other freshwater teleosts, the motility of cyprinid sperm is triggered by exposure to hypo-osmotic media and is inhibited by the high osmolality present in the seminal plasma (230–282 mOsmol kg^{-1}) [34]. The ATP concentration decreases rapidly during the first 30 s after initiation of sperm motility in an activation medium [3]. Moreover, ATP and cAMP concentrations [12], concentrations of ions, especially Ca^{2+} [16], sperm pH [29] and extracellular factors such as temperature [2] and extracellular pH of the medium [15] may affect the capacity of sperm motility and its duration.

The precipitous decline of olive barb abundance in the wild is of great concern among conservation specialists and has made the development of cryopreservation protocols imperative in this species. The present study was aimed at development of cryopreservation techniques for olive barb spermatozoa to use in artificial propagation. The objectives were to: (1) characterize sperm motility and duration; (2) evaluate sperm motility at different osmotic pressures; (3) evaluate the toxicity of cryoprotectants; (4) evaluate the post-thaw motility at different concentrations of two cryoprotectants (DMSO and methanol), and (5) evaluate the fertilization and hatching rates of eggs fertilized using cryopreserved and fresh sperm. To our knowledge, this is the first report on cryopreservation of olive barb sperm.

Materials and methods

Collection and rearing of broods

Mature *P. sarana* (females showing swollen bellies and milt flowing freely from males with gentle abdominal pressure) were collected from two natural sources: the Chalan *beel* under the Natore district, and the Mogra river of the Netrokona district in January of 2008. Fish were transported to brood-rearing earthen ponds (800 m^2) of the Fisheries Field Laboratory complex, Bangladesh Agricultural University (BAU) in Mymensingh and reared for 1.5 years. Water quality parameters including temperature (30.16 ± 2.0 °C), pH (7.58 ± 0.52), dissolved oxygen (5.70 ± 0.55 mg l^{-1}) and total alkalinity (87.48 ± 26.19 mg l^{-1}) of the ponds during reproduction were recorded. The brood fish were fed twice daily a commercial diet (35% protein; Paragon Feeds Limited, Bangladesh) at 4–5% of body weight in the rearing ponds.

Sperm collection

Mature male fishes were collected from the brood-rearing ponds during the breeding season (May–July 2009). After measuring the total length (cm) and body weight (g), the fish were held for as long as 12 h for conditioning in the tanks of the hatchery of the Faculty of Fisheries, BAU. They were injected with carp pituitary supernatant (collected and processed by local fish hatchery owners) at 2 mg kg^{-1} of body weight and released in the conditioning tank. Six hours after injection, the males were captured from the tank and were laid on foam to wipe the urogenital pore. Gentle pressure was applied at the base of the pelvic fin, and sperm were collected in glass vials containing Alsever's solution (0.7% NaCl, 0.8% sodium citrate dissolved in 100 ml water; pH 7.2) prepared at 296 mOsmol kg^{-1}. The samples were placed on ice (4 °C) and brought to the Laboratory of Fish Biodiversity and Conservation, BAU.

Sperm concentration was determined in triplicate counts by use of haemacytometer and expressed as the number of cells $\times 10^9$ ml^{-1}. Milt was diluted 4000-fold in distilled water, and a droplet of the diluted milt was placed within a haemacytometer (area of the smallest square = 1/400 mm^2, depth 0.1 mm) with cover slip (Essential Scientific Bangladesh; made by Marienfeld, Germany). The number of spermatozoa in five large squares (area mm^2) of the counting chamber was counted at 400× magnification.

Sperm quality

Before cryopreservation, the motility of sperm samples was evaluated by use of a light microscope (Novex K-range, Holland) at 400× magnification. Sperm motility was estimated by adding 19 μl of distilled water (24 mOsmol kg^{-1}) as activating medium to 1 μl of fresh milt on a glass slide. The motility was observed within 3–4 s after activation and was expressed as the percentage of sperm that had active forward movement. Sperm vibrating in place without forward movement were not considered to be motile. Only sperm samples showing motility above 80%, were used for cryopreservation experiments.

Experiment 1: motility characteristics

Sperm motility and duration were characterized by using sperm samples ($\geqslant$90% motility) from five fish (replicated in triplicate) after activation with distilled water. Motility was estimated at 5-s intervals until all motility in the sample ceased (no motility, some vibrating in place).

Experiment 2: sperm motility in various osmolalities of sodium chloride (NaCl)

Sperm samples were exposed to 10 graded dilutions of NaCl ranging in osmotic pressure from 48 to 319 mOsmol kg^{-1} to evaluate the relationship between osmotic pressure and sperm motility. The motility was estimated as described above.

Experiment 3: determination of cryoprotectant toxicity

Cryoprotectant concentrations were prepared according to the percentage (5%, 10%, and 15%) in the final suspension with five replicates. Either DMSO or methanol was added with Alsever's solution and further mixed with fresh spermatozoa to yield the final concentration of the cryoprotectant in the suspension. The toxicity of these cryoprotectants was evaluated by observing sperm motility at exposure times of 10, 20 and 30 min.

Experiment 4: evaluation of post-thaw motility of DMSO and methanol

Following the toxicity experiments, cryoprotectant concentrations were chosen and post-thaw motility was evaluated for 5%

and 10% DMSO, and for 5%, 10% and 15% methanol. The cryoprotectants were mixed in Alsever's solution and added to the samples to yield the desired final concentrations (1:1:8 = sperm:cryoprotectant:extender). The sperm samples were equilibrated for 8–10 min for 10% DMSO and 25–30 min for 5% DMSO and 5%, 10% and 15% methanol before freezing. Percent motility was observed for all samples before addition of cryoprotectant and again just prior to the initiation of freezing. Pre-labeled 0.25-ml French plastic straws (Minitüb System, Minitüb, Tiefenbach, Germany) were filled with 0.23 ml of diluted spermatozoa and sealed manually using a heated crucible tongs. After sealing, the straws were transferred to a controlled-rate freezer (FREEZE CONTROL® CL-3300; Australia) programmable by computer-based software (CryoGenesis™ V5). A one-step freezing protocol (5 °C to −80 °C at a rate of 10 °C/min) was used. After freezing, the straws were immediately plunged into liquid nitrogen (−196 °C) for storage of 7–15 days. Straws were removed from the liquid nitrogen and thawed at 40 °C for 7 s in water bath [39] and post-thaw motility of the sperm was assessed within 3–4 s after activation with distilled water as described above.

Experiment 5: assessment of fertilization and hatching

Three fertilization trials were conducted to evaluate the fertility and hatching rates of fresh and cryopreserved spermatozoa. Female olive barb were injected twice with locally available carp pituitary supernatant (6 mg kg^{-1} of body weight; 1/3 of volume as first dose, and 2/3 as second dose after a 6 h interval) and were stripped 12 h after the second injection. Eggs collected from three females were immediately fertilized at room temperature (25 °C) using fresh and pooled cryopreserved spermatozoa (Alsever's solution with 10% DMSO chosen based on the results of "Experiment 4").

After thawing, sperm motility was estimated and 15 straws (2.3×10^9 cells) of cryopreserved sperm were used for fertilization of 2 ml of eggs (1 ml ≈ 5646 eggs). The cryopreserved spermatozoa were activated with tap water (31 mOsmol kg^{-1}). The spermatozoa and the eggs were mixed with a hen feather and placed in hatching jars (radius 0.3 m, height 0.7 m, capacity 90 L) with 12–14 L/min of water exchange used for incubation of eggs. The fertilization rates were calculated as the percentage of fertilized eggs obtained from the total number of eggs at 22–24 h after fertilization. The hatching rates were calculated as the percentage of larvae obtained from total number of eggs for cryopreserved or fresh sperm.

Statistical analysis

All percent motility values were subjected to arcsine transformation prior to statistical analysis. Treatment effects were analyzed using one factor, two-factor or repeated measures ANOVA. Means were separated by Duncan's Multiple Range Test and a value of $P < 0.05$ was considered as being statistically significant. Data were expressed as mean ± SD and analyzed by Statistical Package for the Social Science (SPSS v 11.5). We did not attempt to standardize the sperm concentrations, so no statistical comparisons were made for fresh and cryopreserved samples. The fresh samples were used to evaluate the quality (fertilization and hatch potential) of the eggs.

Results

Sperm quality

The weight of male fish ($N = 9$) ranged between 110 and 140 g (mean ± SD 124.4 ± 9.9 g) and total length ranged between 15 and 22 cm (19.2 ± 2.6 cm). The collected sperm volume varied across individuals from 0.15 to 1.2 ml (0.76 ± 0.29 ml). The sperm concentration ranged from 5.2×10^9 to 7.9×10^9 ml^{-1} ($6.7 \pm 0.8 \times 10^9$ ml^{-1}). Fresh sperm motility was estimated to be from 85% to 95% (89% ± 3%).

Motility characteristics

Sperm attained maximum motility (90%) within 15 s (Fig. 1), when activated with distilled water. Subsequently, the motility percentage decreased significantly ($P = 0.001$) after showing maximal motility until all motility ceased within 35 s after activation.

Activation of sperm motility in different osmolalities

Activation of fresh sperm motility decreased with the increasing osmolalities of NaCl (Fig. 2). Sperm motility was completely inhibited at or above 287 mOsmol kg^{-1}. The highest motility was observed at osmolalities of 128 mOsmol kg^{-1} and below and was considered to be complete activation. The threshold point of motility activation was considered to be 255 mOsmol kg^{-1} (Fig. 2). There was a significant difference between the osmotic pressures at threshold and complete activation ($P = 0.001$).

Toxicity evaluation

The motility of fresh sperm before equilibration with cryoprotectants was 90–95%. In the presence of 5% DMSO sperm retained the capacity for motility activation for 90 min although motility significantly decreased ($P = 0.013$) and completely ceased within 25 min at 10% DMSO. After 5 min of exposure to 15% DMSO motility was reduced to 20% ($P = 0.001$) and ceased completely within 15 min (Fig. 3, top). This motility pattern was similar when sperm were equilibrated with 5%, 10% and 15% of methanol. During 90 min of observation, motility decreased significantly ($P = 0.021$) although more than 60% of spermatozoa were motile when activated at 90 min (Fig. 3, bottom). For fresh sperm held without addition of cryoprotectants, the motility after activation was unchanged (90–95%) after being held on ice for 90 min.

Evaluation of post-thaw motility of DMSO and methanol

Motility of fresh sperm was more than 90% in all cases. After addition of cryoprotectants, motility of the equilibrated spermatozoa was more than 80% although 10% DMSO showed 70% motility (Table 1). Post-thaw motility was significantly affected by cryoprotectant ($P = 0.049$) and its concentration ($P = 0.000$) although post-thaw motility was not affected by their interaction ($P = 0.716$). The

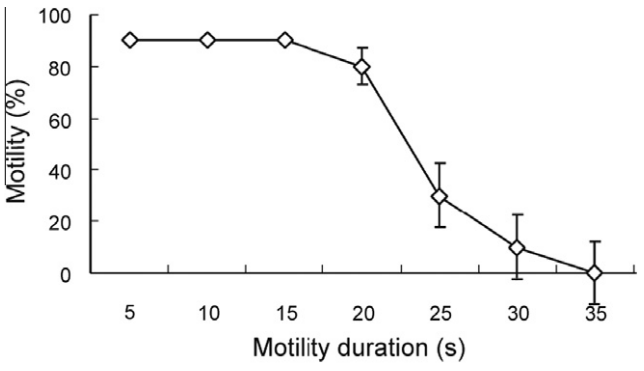

Fig. 1. Motility of olive barb sperm activated with distilled water (24 mOsmol kg^{-1}). Each point represents the mean value (±SD) of sperm samples from four males.

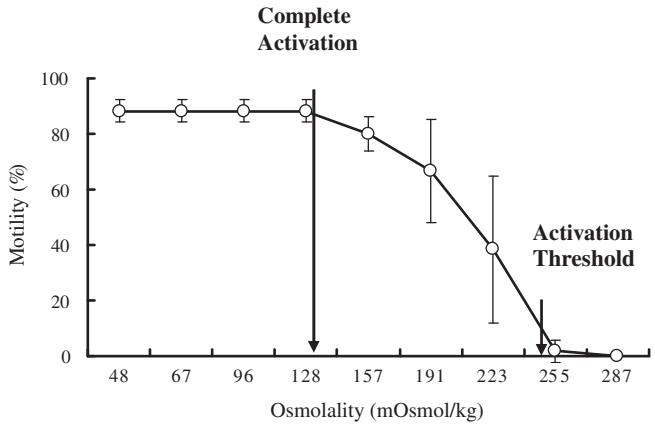

Fig. 2. The motility of olive barb sperm exposed to NaCl solutions prepared at graded osmotic pressures. Each point represents the mean value (±SD) of sperm samples from five males.

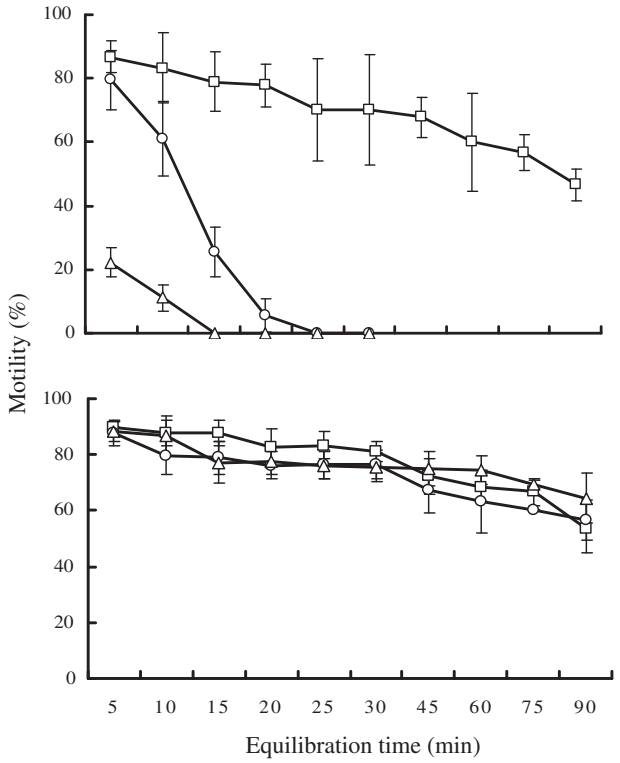

Fig. 3. The toxicity of two different cryoprotectants, DMSO (top) and methanol (bottom) at final concentrations of 5% (squares), 10% (circles), and 15% (triangles) when equilibrated with sperm from olive barb in modified Alsever's solution at an osmolality of 296 mOsmol kg^{-1}. Each point represents the mean ± SD of three males.

sperm samples that contained 10% DMSO had significantly higher post-thaw motility (61 ± 8%) than did any other concentration of DMSO (5%) or methanol (5%, 10% and 15%) (Table 1).

Fertilization trials

When freshly collected eggs were fertilized with fresh sperm, fertilization was 72% and hatching was 62%. After being cryopreserved (Alsever's solution with 10% DMSO chosen based on the results of "Experiment 4") and kept in liquid nitrogen for 15 days, the mean fertilization was 70% and hatching was 37% for thawed sperm with similar batches of eggs. The mean post-thaw motility of sperm was 63 ± 4% when tested with fresh eggs. Although the few fish hatched from cryopreserved sperm, they were active and in as good physical shape as those produced from fresh sperm.

Discussion

Spermatozoa concentration is one of the main indicators of sperm quality [43]. The concentration should be balanced against the optimum cryoprotectant percentage as well as milt dilution ratio in extender. In this study, the concentration of spermatozoa of olive barb was $6.7 \pm 0.8 \times 10^9$ ml^{-1}. Cryopreservation and fry production under farming conditions in silver carp (*Hypophthalmichthys molitrix*) has been possible at a sperm concentration of $1.2 \pm 0.5 \times 10^{10}$ ml^{-1} [4].

After activation in a hypo-osmotic solution, fresh sperm of most fishes show their active forward movement for less than a min [3]. The duration of sperm motility in olive barb was brief (35 s) compared with that of some other freshwater fishes such as muskellunge *Esox masquinongy* (6–7 min) [32] and paddlefish *Polyodon spathula* (4–5 min) [33], but similar to the duration of sperm motility in walleye *Sander vitreus* (51 s) [40], Colorado pikeminnow *Ptychocheilus lucius* (57 s) [48] and razorback sucker (70 s) [46]. The maximal sperm activity of the olive barb was also brief (15 s). When the short duration of motility and varying motility characteristics at different phases after activation, dilution becomes a key issue because volume of the diluent determines the dynamics of sperm activation [7]. As the duration of motility can be important for egg–sperm interaction through fertilization in artificial breeding [53], care should be taken to ensure the complete, early mixing of gametes during artificial breeding of olive barb.

The activation of sperm of freshwater fishes is typically associated with a decrease in osmotic pressure [48]. The motility of olive barb sperm decreased as the osmolality of the extender solution increased, and motility was completely inhibited at 287 mOsmol kg^{-1} and above. The seminal plasma osmolalities of most cyprinids (freshwater fish) are within 230–346 mOsmol kg^{-1} [2]. Therefore, safe storage requires an extender solution with an osmolality similar to that of seminal plasma that could be useful in cryopreservation. Alsever's solution formulated at 296 mOsm kg^{-1} presumably raised the osmotic pressure of diluted sperm and appeared to be effective for protection of sperm during storage.

Cryoprotectants are essential for protection against freezing damage of sperm due to intracellular ice crystal formation [53]. There is no universal cryoprotectant or concentration for cryopreservation of fish sperm [19] and suitable cryoprotectants vary among fish species and studies [50]. This is due to the difference in permeability of cryoprotectants and varying toxicity tolerance level of sperm to the exposure of cryoprotectants [8]. The efficacy of cryoprotectants, however, depends on a delicate balance between toxicity and the capacity to protect the cells [29]. In the toxicity experiment, sperm equilibrated with 5% DMSO and 5% methanol remained motile for a longer duration than those at higher concentrations of cryoprotectants. Because marked differences were observed in pre-freeze and post-thaw motility of 5% cryoprotectants, it appears that this concentration was not toxic, but failed to protect sperm during freezing and thawing. This is an agreement with the result of red snapper (*Lutjanus argentimaculatus*) in which sperm suspended 5% DMSO or methanol were found to survive longer than in 10% DMSO or methanol after equilibration, and produced poorer post-thaw motility after cryopreservation [50]. Dimethyl sulfoxide permeates cells quickly and brings a rapid balance between the intracellular and extracellular fluid concentrations [14]. When cryopreserved with 10% DMSO, a 5–10 min equilibration period provided similar pre-freeze (>70%) and post-thaw (>60%) motility. The result indicates that the cells survive the freezing process, and retain the ability to for progres-

Table 1
Percent sperm motility (mean ± SD; minimum–maximum) from individual olive barb, *Puntius sarana* ($N = 4$) before and after cryopreservation using different concentrations of DMSO and methanol as cryoprotectants with a cooling rate of 10 °C/min from 5 to −80 °C (equilibration time of 5–10 min for 10% DMSO, and 25–30 min for 5% DMSO, 5%, 10% and 15% methanol).

Cryoprotectant	Concentration (%)	Equilibration motility	Post-thaw motility
DMSO	5	85 ± 9% (60–90%)	7 ± 7% (0–20%)
	10	70 ± 14% (40–90%)	61 ± 8% (50–80%)
Methanol	5	89 ± 3% (80–90%)	5 ± 6% (0–20%)
	10	84 ± 7% (70–90%)	53 ± 10% (30–70%)
	15	88 ± 6% (70–90%)	26 ± 11% (10–50%)

sive movement after thawing. Therefore, 10% DMSO (with 5–10 min of equilibration) could be the best option for sperm cryopreservation of olive barb at a cooling rate of 10 °C/min. This corresponds with previous observations on yellowfin sea bream, *Acanthopagrus latus* [18] and yellow perch (*Perca flavescens*) [14]. In the present study, when equilibrated with 15% DMSO, sperm lost the capacity for motility within 15 min. The result suggests that this concentration (15%) was toxic to sperm and unsuitable for cryopreservation of olive barb. High concentrations of DMSO are toxic to fish spermatozoa compared with other cryoprotectants such as methanol, ethanol, and glycerol.

Although different concentrations of DMSO had different effects on sperm viability, methanol was found to be surprisingly non-toxic at all concentrations tested. This result is consistent with that in channel catfish (*Ictalurus punctatus*) where storage of sperm in 5% methanol was surprisingly non-toxic as the samples survived for as long as 5 days prior to freezing [13]. In the present study, spermatozoa cryopreserved in 10% methanol had the highest motility. It provided greater protection to spermatozoa during cryopreservation than did 5% and 15% concentrations. Similarly, 10% methanol gave a high percentage of motility for *Mystus nemurus* sperm [35]. In addition, 10% methanol was the most effective cryoprotectant for common carp [23,21] zebrafish and other fish species including salmonids [30] and catfishes [45].

Post-thaw sperm motility is one of the most important indicators of the success of a cryopreservation protocol if the morphology of the preserved sperm remains unchanged [1]. However, a more appropriate criterion for assessing protocols, when possible, is the ability of the cryopreserved spermatozoa to fertilize eggs [22]. To evaluate the egg quality in this study fresh sperm were mixed with eggs producing 72% fertilization and 62% hatching in which fresh and cryopreserved sperm numbers were not standardized. The fertilization was 70% and hatching was 37% for cryopreserved sperm. Although unknown, a probable cause of this discrepancy in hatching is that eggs were fertilized with thawed sperm and initial development occurred, but further development stopped when complex embryonic differentiation processes began [31]. If so, increasing the number of cryopreserved spermatozoa could increase fertilization of eggs, but deteriorated genetic value due to freezing and thawing or toxicity of diluents, could disrupt or prevent embryonic development [26,4]. This could be the cause of lower hatching of the eggs fertilized with cryopreserved spermatozoa of olive barb compared to that of with fresh spermatozoa. In any event, these results demonstrate that eggs fertilized with cryopreserved sperm can produce viable offspring and it is available for use with threatened species like olive barb where few other options are available to produce progeny in the future. Despite the preceding discussion, it does remain possible that the lower hatching rates of cryopreserved sperm might be compensated for by using higher sperm-to-egg ratios. Thus, it would be necessary to determine the egg-to-sperm ratio (for fresh and cryopreserved sperm) for making direct comparisons of fertilization and hatching rate. In this study the fresh sperm were used only to evaluate the quality of the eggs (which can be variable). The fertilization and hatching rate can be affected by sperm quality, sperm collection procedures, egg quality, extender composition, and cryopreservation procedures including thawing rates [1]. Therefore, further research should address the effects of these factors on successful fertilization, hatch and development.

Conclusion

This study developed a simplified cryopreservation protocol for use with the critically endangered olive barb. In summary, spermatozoa of olive barb could be cryopreserved using Alsever's solution as extender with 10% DMSO (5–10 min equilibration) with a cooling rate of 10 °C/min from 5 to −80 °C.

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References

[1] E. Akcay, Y.S.S.E. Bozkurt, N. Tekun, Cryopreservation of Mirror Carp Semen, Turk. J. Vet. Anim. Sci. 28 (2004) 837–843.
[2] S.M.H. Alavi, J. Cosson, Sperm motility in fishes: (I) effects of temperature and pH, Cell Biol. Int. 29 (2005) 101–110.
[3] S.M.H. Alavi, J. Cosson, Sperm motility in fishes. (II) Effects of ions and osmolality: a review, Cell Biol. Int. 30 (2006) 1–14.
[4] B. Alvarez, R. Fuentes, R. Pimentel, Z. Abad, E. Cabrera, E. Pimentel, A. Arena, High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions, Aquaculture 220 (2003) 195–201.
[5] I. Babiak, J. Glogowski, K. Goryczko, S. Dobosz, H. Kuzminski, J. Strzezek, W. Demianowicz, Effect of extender composition and equilibration time on fertilization ability and enzymatic activity of rainbow trout cryopreserved spermatozoa, Theriogenology 56 (2001) 177–192.
[6] IUCN Bangladesh, Red Book of Threatened Fishes of Bangladesh, IUCN – The World Conservation Union, 2000, p. 116.
[7] R. Billard, M.P. Cosson, Some problems related to the assessment of sperm motility in freshwater fish, J. Exp. Zool. 261 (1992) 122–131.
[8] E. Cabrita, V. Robles, O. Cherguinni, J.C. Wallace, M.P. Herraez, Effect of different cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos (*Scophthalmus maximus*), Cryobiology 47 (3) (2003) 204–213.
[9] E. Cabrita, V. Robles, L. Rebordinos, C. Sarasquete, M.P. Herraez, Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm, Cryobiology 50 (2005) 144–153.
[10] B.K. Chakraborty, M.I. Miah, M.J.A. Mirza, M.A.B. Habib, Rearing and nursing of local Sarpunti, *Puntius sarana*, (Hamilton) at different stocking densities, Pakistan J. Biol. Sci. 6 (2003) 797–800.
[11] J. Chandrasoma, S.S. De Silva, Reproductive biology of *Puntius sarana*, an indigenous species, and *Tilapia rendalli* (melanopleura), an exotic, in an ancient man-made lake in Sri Lanka, Aquacult. Res. 12 (2008) 17–28.
[12] R. Christen, J.L. Gatti, R. Billard, Trout sperm motility: the transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement, Eur. J. Biochem. 166 (1987) 667–671.
[13] J.M. Christensen, T.R. Tiersch, Cryopreservation of channel catfish sperm: effects of cryoprotectant exposure time, cooling rate, thawing conditions, and male-to-male variation, Theriogenology 63 (2005) 2013–2112.
[14] A. Ciereszko, L. Ramseyer, K. Dabrowski, Cryopreservation of yellow perch semen, Prog. Fish Cult. 55 (1993) 261–264.
[15] J. Cosson, O. Linhart, Paddlefish *Polyodon spathula* spermatozoa: effects of potassium and pH on motility, Folia Zool. 45 (1996) 364–370.
[16] M.P. Cosson, R. Billard, L. Letellier, Rise of internal Ca^{2+} accompanies the initiation of trout sperm motility, Cell Motil. Cytoskelet. 14 (1989) 424–434.
[17] R.K. Gallant, G.F. Richardson, M.A. McNiven, Comparison of different extender for the cryopreservation of Atlantic salmon spermatozoa, Theriogenology 40 (1993) 479–486.
[18] J.C. Gwo, Cryopreservation of yellow fin seabream (*Acanthopagrus latus*) spermatozoa (Teleost, Perciformes, Sparidae), Theriogenology 41 (1994) 989–1004.
[19] J.C. Gwo, Cryopreservation of sperm of some marine fishes, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. 138–160.

[20] B. Harvey, Cryopreservation of *Sarotheroden mossambicus* spermatozoa, Aquaculture 32 (1983) 313–320.
[21] B. Harvey, R.N. Kelley, M.J. Ashwood-Smith, Cryopreservation of zebra fish spermatozoa using methanol, Canadian J. Zool. 60 (1982) 1867–1870.
[22] D.C. Honeyfield, W.F. Krise, Measurement of milt quality and factors affecting viability of fish spermatozoa, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. 49–58.
[23] A. Horvath, E. Miskolczi, B. Urbanyi, Cryopreservation of common carp sperm, Aquat. Living Resour. 16 (2003) 457–460.
[24] J.K. Jena, P.C. Das, R. Das, S. Mondal, Performance of olive barb, *Puntius sarana* (Hamilton) in fingerling rearing with rohu, *Labeo rohita* (Hamilton) and mrigal, *Cirrhinus mrigala* (Hamilton), Aquaculture 265 (2007) 305–308.
[25] M.G.Q. Khan, A.U. Ahmed, M.S. Islam, M.F.A. Mollah, Effects of extenders and cryoprotectants on spermatozoal motility of silver carp, Mol. Boil. Biotechnol. 3 (1 and 2) (2005) 53–57.
[26] F. Lahnsteiner, Sperm cryopreservation in the salmonidae and the Northern pike, Aquacult. Res. 31 (2000) 245–258.
[27] F. Lahnsteiner, T. Weimann, R.A. Patzner, A uniform method for cryopreservation of semen of the salmonid fishes *Oncorhynchus mykiss* (Walbaum), *Salmo trutta* f. *fario* L., *Salmo trutta* f. *lacustris* L., *Coregonus* sp., Aquacult. Res. 26 (11) (1995) 801–807.
[28] F. Lahnsteiner, R. Patzner, T. Wiesmann, Semen cryopreservation of salmonid fish: influence of handling parameters on the post-thaw fertilization rate, Aquacult. Res. 27 (1996) 659–671.
[29] F. Lahnsteiner, B. Berger, T. Weismann, R.A. Patzner, The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation, J. Appl. Ichthyol. 12 (1996) 99–106.
[30] F. Lahnsteiner, T. Weismann, R.A. Patzner, Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes, Aquacult. Res. 28 (1997) 471–479.
[31] F. Lahnsteiner, B. Berger, T. Weismann, Effects of media, fertilization technique, extender, straw volume, and sperm to egg ratio on hatchability of cyprinid embryos, using cryopreserved semen, Theriogenology 60 (2003) 829–841.
[32] F. Lin, K. Dabrowski, Effects of sperm irradiation and heat shock on induction of gynogenesis in muskellunge (*Esox masquinongy*), Canadian J. Fish Aquat. Sci. 53 (1996) 2067–2075.
[33] S.D. Mims, Paddlefish: an aquacultural species?, Farm Pond Harvest 24 (1991) 18–20
[34] M. Morisawa, S. Oda, M. Yoshida, H. Takai, Transmembrane signal transduction for the regulation of sperm motility in fishes and ascidians, in: C. Gagnon (Ed.), The Male Gamete: From Basic Knowledge to Clinical Applications, Cache River Press, Vienna, USA, 1999, pp. 149–160.
[35] Z.A. Muchlisin, Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation, Biodiversitas 6 (1) (2005) 66–69.
[36] M. Mukherjee, A. Praharaj, S. Das, Conservation of endangered fish stocks through artificial propagation and larval rearing technique in West Bengal, India, Aquac. Asia 7 (2002) 8–11.
[37] A.K.A. Rahman, Freshwater Fishes of Bangladesh, second ed., Zoological Society of Bangladesh, Dhaka, Bangladesh, 2005. p. 394.
[38] G.F. Richardson, T.L. Miller, M.A. McNiven, Cryopreservation of Arctic charr, *Salvelinus alpinus* L., semen in various extenders and in three sizes of straw, Aquaculture 31 (2000) 307–315.
[39] L.K. Riley, G.C. Holladay, J.E. Chesney, T.R. Tiersch, Cryopreservation of sperm of red snapper (*Lutjanus campechanus*), Aquaculture 238 (2004) 183–194.
[40] J.R. Satterfield, S.A. Flickinger, Field collection and short-term storage of walleye semen, Prog. Fish Culturist. 57 (1995) 182–187.
[41] M. Sinha, Observations on the biology of *Puntius sarana* (Hamilton) of Loni Reservior; length–weight relationship, food and condition factor, J. Inland Fish Soc. 4 (1973) 122–131.
[42] J. Stoss, W. Holtz, Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm: effect of proteins in the diluent sperm from different males and interval between sperm collection and freezing, Aquaculture 31 (1983) 275–282.
[43] M. Suquet, M.H. Omnes, Y. Normant, C. Fauvel, Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*), Aquaculture 101 (1992) 177–185.
[44] T.R. Tiersch, Introduction, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. xix–xxvi.
[45] T.R. Tiersch, C.A. Goudie, G.J. Carmichael, Cryopreservation of channel catfish sperm, storage in cryoprotectants, fertilization trial, and growth of channel catfish produced with cryopreserved sperm, Trans. Am. Fish Soc. 123 (4) (1994) 580–586.
[46] T.R. Tiersch, W.R. Wayman, C.R. Figiel, O.T. Gorman, J.H. Williamson, G.J. Carmichael, Field collection, handling and storage of sperm of the endangered razorback sucker, N. Am. J. Fish Man. 17 (1997) 167–173.
[47] T.R. Tiersch, C.R. Figiel, W.R. Wayman, J.H. Williamson, G.J. Carmichael, T.O. Gorman, Cryopreservation of sperm of the endangered razorback sucker, Trans. Am. Fish Soc. 127 (1998) 95–104.
[48] T.R. Tiersch, C.R. Figiel, W.R.J. Wayman, H. Williamson, O.T. Gorman, G.J. Carmichael, Cryopreservation of sperm from the endangered Colorado pikeminnow, N. Am. J. Aquacult. 66 (2004) 8–14.
[49] B. Urbanyi, A. Horvath, Z. Varga, L. Horvath, I. Magyary, F. Radics, Effects of extenders on sperm cryopreservation of African Catfish, *Clarias gariepinus* (Burchell), Aquacult. Res. 30 (1999) 145–151.
[50] V. Vuthiphandchai, S. Chomphuthawach, S. Nimrat, Cryopreservation of red snapper (*Lutjanus argentimaculatus*) sperm: effect of cryoprotectants and cooling rates on sperm motility, sperm viability, and fertilization capacity, Theriogenology 72 (2009) 129–138.
[51] W.R. Wayman, R.G. Thomas, T.R. Tiersch, Cryopreservation of sperm of spotted seatrout (*Cynoscion nebulosus*), Gulf Res. Rep. 9 (3) (1996) 183–188.
[52] P.A. Wheeler, G.H. Thorgaard, Cryopreservation of rainbow trout semen in large straws, Aquaculture 93 (1991) 95–100.
[53] H. Yang, C. Carmichael, Z.M. Varga, T.R. Tiersch, Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*, Theriogenology 68 (2007) 128–136.