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Optimisation of cryovials and milt dilution ratios to upscale carp sperm cryopreservation for use in hatchery seed production

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Abstract

This study reports the experimental results carried out to optimise containers and milt dilution ratios for carp sperm cryopreservation, in order to make the process simple and compatible with the needs of commercial carp seed production, which is a small-scale and low technology sector in Asia. The procedure optimisation for up-scaling was assessed by the ability of cryopreserved sperms to fertilise *Cyprinsu carpio* eggs and hatching percentage, in three different experiments. In the first experiment, we tested the effect of milt to different extender dilutions (1:4, 1:6 and 1:8); the second experiment tested the effect of container capacities (0.5 ml French straw; 2 ml and 5 ml cryovials) with two dilution ratios (1:3 and 1:6) and in the third experiment, the efficacy of pre-mix of extender salts and individually pre-weighed salts stored over 5 months was compared with fresh preparation of extender. Out of the three dilutions tested, two dilutions *viz.,* 1:4 and 1:6, yielded hatching above 50%. We also used the milt cryopreserved at dilution of 1:3 which was post-thaw diluted with extender, to make final dilution of 1:6, to fertilise the eggs. This post-thaw dilution in the ratio 1:6, yielded the results comparable to the milt cryopreserved in 1:6 dilution. This approach can help small-scale seed producers to store more sperm within the limits of the cost of liquid nitrogen. The extender salt composition, pre-weighed and pre-mixed, was also found to give comparable results to the fresh composition after storage over 5 months. This can assist in developing ready to use, cost-effective, working kits available to semi-technical hatchery seed producers. The parameters optimised in the study has potential to be transformed as an easy strategy with the use of 5 ml vial, cryopreserved at 1:3 dilution, with further post-thaw dilution at site, thus aiding in fertilisation of large volume of eggs produced by high fecund carp species within the limited time available for maintaining good gamete quality.

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Introduction

Protocols for fish sperm cryopreservation have been tested for over 200 fish species (Tiersch and Green, 2011, Tsai and Lin, 2012, Diwan *et al.,* 2020). The potential utility of this technology in aquaculture for cross-breeding, facilitating genetic exchange and for conservation of precious germplasm is well accepted (Diwan *et al.,* 2010; Tsai and Lin, 2012) especially in salmonids (Sylwia, 2019; Francky, 2021). However, there are still not many examples of large-scale application of this technique in commercial fish seed production for the fish species except for some experimental reports (Tiersch and Green, 2011; Varkonyi, 2019). Protocol for commercial-scale cryopreservation of Eurasian perch sperm collected off-season was successfully developed and optimised by Bernath *et al.* (2016a). While working on hybridisation, Hu *et al.* (2011, 2014) conducted high-throughput cryopreservation of blue catfish sperm at an industrial level to produce hybrid catfish. Overcoming some biological and procedural bottlenecks pose challenge to get consistent results from the technique, in terms of quality and quantity, which is the primary need for

any commercial use. The optimum conditions required for sperm cryofreezing can vary significantly, even among the closely related species and therefore, development and validation of species-specific protocols is imperative.

Common carp *Cyprinus carpio* (Linnaeus, 1758) has been introduced in many countries for aquaculture and is an important source of low-cost protein contributing nearly 4.23 million t to global production (FAO, 2022). With the available selective bred varieties such as Amur carp (India), FFRC strain (China) and Hungarian strain, common carp culture is popular in diversified climatic regions. Common carp has been used as a model fish to study sperm cryopreservation (Kurokura *et al.,* 1984, Lahnsteiner *et al.,* 2000; Linhart *et al.,* 2003; Irawan *et al.,* 2010; Yavas *et al.,* 2014; Bernath *et al.,* 2016b; Sharma *et al.,* 2017; Levente, 2018). Carps are high fecund species and most hatcheries fertilise eggs from many brooders simultaneously and therefore, sperm should be available in optimum volume and used within the limited processing time, to achieve quality fertilisation and hatching. To make use of the potential of sperm cryopreservation in carp aquaculture, the research findings need to be transformed into simple tools so that technology is compatible to commercial aquaculture for cost and effort of the small-scale rural seed producers. There is significant scope to substitute the expensive laboratory-based assessment procedures with more efficient field-based methodologies that have been meticulously calibrated to align with established laboratory protocols. Such a transition holds promise for attracting interest from semi-technical seed producers in Asia, who might not have otherwise readily adopted the technology. Presently, this technology remains primarily confined to a select high value species in developed nations. An essential requirement in this context is the precise control of spermatozoa concentration in milt which is a prerequisite to ensure consistent and reproducible results for cryopreservation and *in vitro* fertilisation protocols (Yang *et al.,* 2016).

Limited progress has been made to preserve the milt in large quantity using cryovials. Cryopreserving large quantity of fish milt will have implications for commercial aquaculture, mainly to produce genetically diversified fish seed. In most research, only 0.25 and 0.5 ml straws, normally used in livestock, were used for milt storage (Viveiros and Godinho, 2009). The straws are widely used; however, such containers have limitations for commercial fish seed production. Cryopreservation using milt containers with larger storage capacity could decrease costs and the space needed for sample storage in cryogenic container, as well as in improving procedures for freezing and thawing milt for artificial fertilisation (Cabrita *et al.,* 2010). Several types of semen containers are commercially available and can be used in sperm cryopreservation, including cryotubes, bags and plastic vials with varied storage capacities (1-10 ml). Cryovials have convenience of easy filling and provide a high surface to volume ratio which facilitates freezing and thawing at uniform rates (Hofmo and Almlid, 1991; Richardson *et al.,* 1999). Use of cryovials has already been observed for cryopreservation in species like common carp (*C. carpio*), Atlantic halibut (*Hippoglossus hippoglossus*), seabass (*Dicentrarchus labrax*), Tambaqui (*Colossoma macropomum*) and medaka (*Oryzias latipes*) (Aoki *et al.,* 1997; Fauvel *et al.,* 1998; Linhart *et al.,* 2000; Horvath *et al.,* 2007; Ding *et al.,* 2011; Maria *et al.,* 2015). Ji *et al.* (2004) and Chen *et al.* (2004) performed cryopreservation in sea perch (*Lateolabrax japonicus*) and turbot (*Scophthalmus maximus*) respectively, using 1.8 ml cryovials. Varkonyi *et al.* (2019) worked on Hungarian carp landrace (*Cyprinus carpio carpio morpha accuminatus*) and used 10 ml cryovials for milt preservation to obtain superior quality sperm during or off-spawning season for the hatcheries. Cryovials can have considerable potential for commercial seed production in highly fecund species such as common carp and Indian major carps. However, as milt container characteristics influence freezing and thawing rates (Kozink *et al.,* 2006) and there are great biological variations among species, studies are needed to develop species specific protocols.

With respect to commercialisation, experiments for up-scaling the storage capacity of cryovials are being conducted as this could minimise the time required for sperm packaging and thawing, reduce the cost and space needed for sample storage in the cryogenic container and facilitate fertilisation of large egg batches from high fecund species like carps. In this background, here we evaluated the effect of extender, dilution, cryovial capacity and use of extender premix on the quality and fertilisation capability of cryopreserved common carp milt.

Materials and methods

Fish handling

This study was approved by the Animal Ethics Committee of National Bureau of Fish Genetic Resources. All animals used in this study were handled according to the guidelines for animal experimentation. The work presented in this study was conducted during 2020 and 2021. *Cyprinus carpio* var. communis were procured locally from the Live gene bank of ICAR-National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow. Fishes were maintained in standard recommended feeding regime and water quality. Sexually mature brooders (N=50, weighing 600-1500 g) were selected for hormonal induction during the breeding season (Feb-March). Hormonal induction was performed *via* intraperitoneal injection (male: 0.2 ml kg^{-1} body weight) of commercial gonadotropin GnRH (Ovarim) to increase milt volume and decrease viscosity, as a routine method for cyprinids. For administration of hormonal dose, fishes were anaesthetised using 2-Phenoxyethanol (0.4 ml l^{-1}) as per Bernath *et al.* (2016b). For hormonal induction of females, intraperitoneal injections were administered at 0.5 ml kg^{-1} body weight.

Collection of milt

Live, running ripe milters of common carp were used for milt collection. After 4 h of hormonal induction, at a water temperature of 27±1°C, the urogenital papilla was carefully wiped to avoid activation or contamination of water, blood, urine and faeces. Milt was collected in dry plastic boxes by applying abdominal pressure in anaesthetised condition. For each male, separate milt box was used for milt collection with proper labeling. The milt boxes were stored in an insulated container on crushed ice without direct contact. Insulated container was transported to the sperm cryopreservation laboratory.

Evaluation of sperm motility

Sperm motility was assessed on microscopic slide under compound microscope (x400). Small quantity of fresh milt (1 µl) was taken on slide and covered with coverslip and observed. If spermatozoa were found motile, sample was discarded. Samples with immotile spermatozoa were activated by adding 10 µl of distilled water. Samples with visual motility of above 70% were selected for cryopreservation.

Quantification of spermatozoa

Spermatozoa concentration in fresh milt samples was determined by hemocytometer counts. Neutral buffered formalin (NBF 10%) with tinge of Eosin was used to dilute and fix the milt to prevent movement before loading. From each box, 100 µl of fresh milt was added in 400 µl NBF solution for quantification of spermatozoa. Serial dilution was used to dilute milt sufficiently so that counting becomes easy. Cells were counted in all 4 big corner squares and mean value was taken for sperm density calculation using the formula: Spermatozoa per ml = Total no. spermatozoa/4 x dilution factor x 10,000. Mean sperm density was estimated by taking samples from pooled milt.

Cryopreservation of milt

Extender with code No. '7' (NaCl 750 mg, KCl 20 mg, $CaCl_2$ 20 mg and $NaHCO_3$ 20 mg dissolved in 100 ml deionised water), suggested by Kurokura *et al*. (1984) for common carp and another extender composition designated '9C' (NaCl 650 mg, KCl 141 mg, $CaCl_2$ 30 mg, $MgSO_4.7H_2O$ 20 mg and glucose 54 mg dissolved in 100 ml deionised water) were used in this study. In experiment 1 and 3, only extender 7 was used, while in experiment 2, two different lots of milt were cryopreserved in extender 7 and extender 9C. The extender solution was stored in chilled condition on crushed ice in an icebox. Ten percent (v/v) dimethyl sulphoxide (DMSO; HIMEDIA Laboratories Pvt. Ltd., Mumbai) was used as cryoprotectant. After quality assessment, all pooled milt was diluted with extender and DMSO was added to the extender just prior to mixing with milt. The milt: extender: DMSO ratios used were, 1: 2.6: 0.4; 1: 3.5: 0.5; 1: 5.3: 0.7 and 1: 7.1: 0.9 in 1: 3, 1: 4, 1: 6 and 1: 8 dilutions respectively. French medium straws (Minitubes, Gmbh, Germany), 2 ml and 5 ml cryovials (Abdos Labtech Pvt. Ltd., New Delhi, India) were used for preserving milt. The extended milt was equilibrated over ice for 10 min which included the time for filling in 0.5 ml French medium straws, 2 ml cryovials and 5 ml cryovials. French straws were filled using manual filling system sucking from cotton plugged side and sealed with polyvinyl alcohol (PVA) powder. Cryovials were filled with stepper and tightly screw capped. After equilibration, straws and cryovials were exposed to liquid nitrogen (LN_2) vapours on a freezing stand at 2.5 cm above surface of LN_2 in Styrofoam box. This method has been calibrated with programmable freezer with rate of cooling -10°C per min. After 10 min of holding in liquid nitrogen vapours, frozen straws and cryovials (-90°C to -100°C) were plunged into liquid nitrogen (-196°C) and stored in dewar flasks as per label for future use.

Fertility trial

Eggs of running ripe females were used for fertility trial and was obtained approximately 12 h after hormonal induction. Eggs were stripped by applying abdominal pressure and collected individually from each female. Eggs from different females were not pooled and stored individually. To thaw frozen milt, the straws and cryovials were rapidly plunged into warm water at 40°C with vigorous shaking and time elapsed was recorded. Food-grade plastic basins (1 l) were used for fertilisation of measured volume of eggs (1 spoonful approximately 9 ml ~ 13,586±925 eggs) and was calculated as 13,500 for all practical purposes. One spoonful of egg was fertilised with 2 ml cryopreserved milt, mixed well with shaking and 5 ml of tap water was added to activate spermatozoa. In control, 300 µl fresh milt was added to one spoonful of egg to ensure equal egg-sperm ratio, mixed well and activated using tap water. From every basin, three replicates of 300 µl of fertilised eggs were taken in three basins (0.5 l) for hatching estimation. Other portion of fertilised eggs were incubated in flow-through incubation system. After washing, eggs turned adhesive, water was exchanged at regular interval of 3 h. Hatching took place between 48 to 56 h after fertilisation. From the total number of egg present in bowl kept for incubation, number of hatchlings was counted to obtain hatching percentage using the formula:

Hatching (%) = (No. of hatchlings / (Dead embryos + Hatchlings) *100)

Experiment 1: Effect of dilution on hatching

Milt of five males were collected separately in different boxes, quality was evaluated and diluted at a ratio of 1: 4, 1: 6 and 1: 8 (V/V) in diluent composed of extender 7 and 10% DMSO (v/v) as cryoprotectant. Pooled milt was divided into equal volumes, diluted in different dilution and filled in 2 ml cryovials, cryopreserved and stored for future use. Four groups *viz*. Control (Fresh milt), Vial 214 (2 ml cryovial and 1: 4 dilution), Vial 216 (2 ml cryovial, 1:6 dilution) and Vial 218 (2 ml cryovial, 1: 8 dilution) were compared. Fertility trial was done using eggs from running ripe females and the hatching percentage was calculated as described above.

Experiment 2: Effect of milt cryopreservation in straws and cryovials on hatching

Milt was collected from fifteen males separately in different boxes and motility test was done individually. All passed samples were pooled and different aliquots were diluted at a ratio of 1:3 and 1:6 (v/v) in diluent composed of extender 7 and 10% DMSO (v/v) as cryoprotectant. Diluted milt was divided into different volumes as per the number of straws, 2 ml vials and 5 ml vials, cryopreserved and stored for future use, which was labelled as Lot_1. Similarly, it was repeated for another set (15 nos.) of males and milt cryopreserved with extender 9C and 10% DMSO was stored as Lot_2 for future fertility trial. Seven groups *viz.*, 1. Control (Fresh milt), 2. Straws13 (0.5 ml straws and 1:3 dilution), 3. Straws16 (0.5 ml straws and 1:6 dilution), 4. Vial 213 (2 ml cryovial, 1:3 dilution and post-thaw dilution with equal quantity of extender), 5. Vial 216 (2 ml cryovial, 1:6 dilution), 6. Vial 513 (5 ml cryovial, 1:3 dilution and post-thaw dilution with equal quantity of extender) and 7. Vial 516 (5 ml cryovial, 1:6 dilution) were compared. For the post-thaw milt onsite dilution treatment (group 2, 4 and 6), only 2 ml of the diluted milt was used in fertility trial to ensure comparable sperm: egg ratio with other treatments. In group 7, only 2 out of 5 ml cryopreserved milt was used. Fertility trial was performed as described above.

Lot_1 and Lot_2 samples were tested in two different days with different sets of females. Eggs from only one female were used in every replicate to minimise effect of egg quality on hatching. These pre-labeled basins were kept at room temperature for incubation and water was exchanged every 3 h. Similarly, Lot_2 cryopreserved milt was compared with fresh milt, using eggs from another set of females. The hatching percentage was estimated as given above, for both the trials.

Experiment 3: Effect of premix, pre-weighed and freshly prepared extender solutions on hatching

Extender solutions with 10% (v/v) DMSO as cryoprotectant was used to cryopreserve carp milt in this experiment. The experiment had a control (fresh milt) and three treatment groups (cryopreserved milt) for comparison; 1. Fresh weighed salts, to prepare stock solution and proportionately mixed to get working extender; 2. Individually pre-weighed salts, stored over 5 months which was used to prepare stock solution and working extender; 3. Premixed salts as per extender composition, stored over 5 months that was used to prepare working extender solution. Milt was cryopreserved with working extender 7 prepared under the three treatments as explained above, and salts were dissolved in 100 ml to get working extender solution. Cryopreservation of milt was done in 2 ml cryovials, 1:6 dilution (milt: extender: DMSO of 1: 5.3: 0.7) using standard protocol of 10 min over ice followed by10 min over liquid nitrogen vapour and thereafter, plunging in liquid nitrogen and stored in cryocan for future fertility trial experiment. Fertility trial was done as described above and hatching percentage were estimated.

Statistical analysis

Statistical analyses were conducted with the IBM SPSS Statistics software ver. 26.0. The distribution of the residuals for each model was tested for normality and an arcsine transformation was performed on the data to achieve a normal distribution. The data were subjected to Levene's test of homogeneity of variance. Wherever, Levene's test was rejected, Welch analysis of variance was applied and difference of means was compared by the Games-Howell test to classify differences between the treatments. Wherever Levene's test failed to reject, classical one-way analysis of variance was applied and means were compared with Tukey's *post-hoc* test. Statistical significance was set at $p<0.05$.

Results

Experiment 1: Effect of dilution on hatching

Complete thawing of 2 ml cryovials took 110 s in 40ºC warm water. Sperm:egg ratio of ~15,00,000 was used in fertility trial *i.e.,* 2.1x1010 spermatozoa were used for 13,500 eggs. Cryomilt preserved in different dilutions were compared with fresh milt. Hatching % recorded were 58.7 (Vial 214), 51.3 (Vial 216), 42.9 (Vial 218) in treatments and 75.4 in control. For the treatment Vial 214 and Vial 216, hatching obtained was above 50%. Hatching percentage relative to fresh milt ranged from 56.9 (Vial 218) to 77.9 (Vial 214). Means were significantly different ($p<0.05$) in analysis of variance (Table 1) between the treatments.

Experiment 2: Effect of straw and vial type on hatching

In the fertility trial, sperm to egg ratio was maintained, similar to experiment 1. Complete thawing of cryopreserved milt in straws (0.5 ml), 2 ml vials and 5 ml vials took 10 s, 110 s, 130 s respectively. Fertility trial was done for Lot_1 and Lot_2 with different sets of females and on different days. However, experiment was done in two lots, data were analysed both lot-wise and combined. Welch analysis of variance was done and difference of means was found statistically significant between the treatments and control ($p<0.05$). There was variation in results of different lots, but when results were compared with respect to control, it showed similar pattern. In combined analysis, hatching percentages of different groups were significantly less ($p<0.05$) than the control (88.4) and it ranged from 47 in Straw13 to 55 in Vial 216 (Table 2). Games-Howell test exhibited significant difference, with all the treatments lower ($p<0.05$) than the control value. However, the treatments did not differ significantly ($p>0.05$). The post-thaw dilution of milt (Vial 2I3 and Vial 513) with extender yielded comparable results to the milt cryopreserved in dilution 1:6 (Vial 216 and Vial 516).

Experiment 3: Effect of premix, pre-weighed and freshly prepared extender on hatching

The sperm:egg ratio mentioned in experiment 1 was followed for experiment 3. Five months old premix of salts and pre-weighed salts of extender 7 were used for cryopreservation and compared with freshly prepared stock solution. Hatching percentage of different groups was less than the control (65.7%) (Table 3) and it ranged from 47% in freshly prepared stock solution to 54.5% in solution prepared with pre-weighed salts. However, there was no significant difference between the three groups. (Table 3).

Discussion

This study on milt cryopreservation of common carp is aimed to facilitate production of genetically diversified fish seed for commercial aquaculture. Here we have used extender reported by Kurokura *et al.* (1984) and 10% DMSO (v/v) as cryoprotectant for milt cryopreservation with 10 min equilibration on crushed ice, 10 min on vapour phase of liquid nitrogen and upon freezing by plunging in liquid nitrogen. Use of straws for semen cryopreservation is appropriate for terrestrial animals, but fecund fishes like common carp may require good volume of cryomilt to achieve maximum fertilisation of eggs. Therefore, use of 5 ml cryovial for milt cryopreservation will be more appropriate for commercial aquaculture as it contains more numbers of spermatozoa. In the present study, time taken

Table 1. Comparison between hatching percentage obtained for different dilution ratios with No. of observations (N). The treatment means with significant differences are indicated by different superscripts (Welch analysis of variance and Games-Howell test)

Treatment (N)	Hatching % (Mean±SD)
Control (3)	75.4 ± 0.8^{a}
Vial214 (3)	58.7 ± 0.2^{b}
Vial216 (3)	51.3 ± 0.2^{bc}
Vial218 (3)	42.9 ± 0.2^{c}

Table 2. Comparison between hatching percentage obtained with lot-wise and combined analysis for different treatments implemented as two replicates with three observations per replicate and total No. of observations (N). The treatment means with significant differences are indicated by different superscripts (Welch analysis of variance and Games-Howell test)

Treatment (N1 and N2)	Lot1 (Mean±SD)	Lot2 (Mean±SD)	Combined (Mean±SD)
Control (6)	92.8±8.9^{a}	83.0±5.2^{a}	88.4±12.9^{a}
Straw13 (6)	49.4±4.4^{c}	44.6±3.3^{c}	47.0±4.4^{c}
Straw16 (6)	48.5±10.8^{c}	49.0±4.2^{c}	48.7±7.8^{c}
Vial213 (6)	56.2±7.2^{b}	43.6±9.4^{c}	50.0±11.0^{b}
Vial216 (6)	50.5±8.4^{b}	60.4±11.1^{b}	55.6±11. 2^{b}
Vial513 (6)	67.6±4.9^{b}	35.1±9.7^{d}	52.3±21.2^{b}
Vial516 (6)	57.6±7.6^{b}	49.0±3.0^{c}	53.4±7.6^{b}

for complete thawing at 40°C with vigorous shaking for straw, 2 ml vials and 5 ml vials was 10, 110 and 130 s respectively. Varkonyi *et al*. (2019) reported thawing of common carp milt cryopreserved in 5 ml straw and 10 ml cryovial in 35 and 240 s at same temperature. Such time difference in thawing may be due to change in material and thickness of straw and cryovial. It is worth mentioning that for cyprinid spermatozoa, thawing temperature should be between 35 to 40°C as in this temperature range, the membrane stability of spermatozoa is maintained efficiently and enzymatic activities are best reactivated (Lahnsteiner *et al.,* 2000). In another experiment, Francis *et al.* (2013) reported highest motility (above 80%) in Indian major carps when thawing was done at 40°C.

Fresh common carp milt had better quality and resulted in higher hatching percentage, while milt stored in cryovials showed comparatively lower hatching percentage, but the difference in hatching percentage was not statistically significant. Previous reports have revealed that the type of container and milt volume directly influence cooling rate and, consequently affect sperm quality of cryopreserved milt (Richardson *et al.,* 1999; Cabrita *et al.,* 2001; Rodina *et al.,* 2007). In this study we have observed that hatching of common carp eggs fertilised with milt derived from 2.0 and 5.0 ml cryovials were better than 0.5 ml straws but not significantly different.

Effect of dilutions (milt to extender)

Cryomilt diluted in extender in the ratios, 1: 4, 1: 6 and 1: 8 were compared with fresh milt and hatching percentage ranged from 42.9 (Vial218) to 75.4 (Control). For the dilutions 1:4 and 1:6, hatching % estimated was above 50. In previous studies, different sperm dilution ratios in common carp have been reported. Boryshpolets *et al.* (2009) reported as low as 1: 1 sperm dilution ratio that resulted in reduced motility (25%). Sharma *et al.* (2017) cryopreserved milt in 1:4 dilutions in two different breeding seasons *viz.* monsoon and post-monsoon and reported varying hatching r of 51-62% and 31-42%, respectively. In another study, Linhart *et al.* (2000) reported sperm dilution of 1:5 and achieved 56±10% fertilisation rate. Similarly, Lahnsteiner *et al.* (2000), diluted sperm in 1:7 ratio and obtained 35 to 65% post-thaw motility of bleak (*Chalcalburnus chalcoides*) spermatozoa. Horvath *et al.* (2003) reported 74±15% fertilisation using 1:9 sperm dilution ratio. However, Bernath *et al.* (2016b), used dilution ratios between 1:5 to 1:20 using sugar-based grayling as extender and observed a reduced fertilisation rate of 32±6%, from thawed sperm that was cryopreserved in 1: 9 dilution ratio compared to control (73±8%). In this study, invariably control was significantly better than all three dilutions of 1:4, 1:6 and 1:8 that we used (Table 2). Nevertheless, dilutions of 1:4 and 1:6 resulted in hatching percentage of 58 and 51 respectively compared to control (75%). Bulk fertilisation using cryomilt diluted in 1:6 ratio will be helpful to hatchery owners for commercial seed production.

Table 3. Comparison between hatching percentage obtained for different treatments implemented as three replicates with three observations per replicate and total No. of observations (N). The treatment means with significant differences are indicated by different superscripts (One-way analysis of variance was applied and means were compared with Tukey's *post-hoc* test)

Treatment (N)	Hatching % (Mean±SD)
Control (9)	65.6±6.3^{a}
Fresh (9)	47.1±4.5^{b}
Pre-weighed (9)	54.5±2.4^{ab}
Premix (3)	47.8±1.5^{b}

Effect of storage containers, straws and vials, on hatching success

In this study, hatching percentages recorded for all the treatment groups were comparable to fresh milt. In both the lots, control milt performed better, however, there was no significant difference between straws and vials used for storage. Thus, it can be inferred that hatching percentage is not affected by the milt storage container. Hatching percentage varied significantly between control milt and milt cryopreserved in straws, 2 and 5 ml cryovials (Table 3). In this study for fertility trial, we had used 300 µl fresh milt to fertilise approximately 13,500 eggs. Approximately 15,00,000 sperm cells derived from cryopreserved milt were used to fertilise one egg. It is worth mentioning that comparison of hatching percentage, without comparing sperm:egg ratio will affect the results significantly (Di Lorio *et al.,* 2019). Thus, in order to make it comparable, sperm:egg ratio is worth mentioning in fertility experiment. Vuthiphandchai *et al.* (2015) used 8,00,000 spermatozoa cells per egg in silver barb (*Barbodes gonionotus*) to achieve hatching of ≥50%. While working with Channel catfish (*Ictalurus punctatus*), Tiersch *et al.* (1994) reported satisfactory fertilisation and hatching percentage using 2,00,000 spermatozoa per egg. Maria *et al.* (2006) reported sperm: egg ratio as high as 46, 00,000 in Piracanjuba (*Brycon orbignynanus*). Warnecke and Pluta (2003) reported sperm egg ratio of 1,00,000 in common carp. In this study, we used 15,00,000 spermatozoa cells to fertilise one egg. Similarly, hatching rate of turbot was not significantly different when ova were inseminated with fresh compared to frozen-thawed sperm (Suquet *et al.,* 1998). However, in our study there was significant reduction in hatching percentage when cryopreserved milt was used. Our observation is very much similar to the results reported by Fauvel *et al.* (1998), who reported that hatching rates of eggs inseminated with frozen-thawed seabass sperm (69%) were significantly lower than those obtained with fresh sperm (81%). Probably the reduction in hatching percentage using cryopreserved milt is due to variation in thawing temperature and incubation method of embryos. Generally, embryos incubated in a flow-through system increases the hatching (Kutluyer *et al.,* 2014; Nynca *et al.,* 2016; Gallego *et al.,* 2017). In this study we could not use flow-through system to incubate

the embryos in replicates as our sample size was too large to accommodate in such system. Nevertheless, in our future study we will validate the previous reports on increase in hatchability using flow-through system. Interestingly, in the present study we did not observe any morphological anomalies in the larvae produced with frozen-thawed spermatozoa and the larvae were similar to control. Similar observation was previously reported in cod species (Mounib, 1978). Generally, the morphological anomalies in fish larvae are attributed to embryo incubation conditions such as temperature, dissolved oxygen and pollution level of water (Boglione *et al.,* 2013). Though, there may be interaction effect of dilution ratio, material of container and volume of vials, analysing such interaction was not aimed in this study. The objective was to elucidate the optimum protocol using vials, as straws are not convenient containers for large-scale fertilisation within limited time period.

Cryopreservation in lower dilution and post-thaw on-site dilution

This experiment has genesis from our experience with hatchery owners, who participated in the ongoing program for validating use of cryopreserved sperm as means for exchange of germplasm and produce seed in commercial hatcheries. For hatchery owners, liquid nitrogen and transport of cryopreserved milt is a recurring cost. This experiment was carried out with an objective to mprove the cost-effectiveness. Here, we cryopreserved common carp milt in 2 ml cryovial (1:3 dilution) and after thawing, equal volume of extender without DMSO was added to make 4 ml milt, thus, we were able to fertilise double volume of eggs. Our observation revealed that, further dilution of milt prior to fertilisation of eggs makes no difference in hatching when compared to milt cryopreserved in 1:6 dilutions. In experiment 2, two extenders 7 and 9C were used for cryopreserving two different lots of milt. Post-thaw dilution has worked well with extender 7 (Lot_1) but in extender 9C (Lot_2), significantly lower hatching % was obtained with vials preserved in the dilution of 1:3. One of the striking advantages of this approach is that a greater number of spermatozoa can be cryopreserved in vials in 1:3 dilution with extender 7. This is crucial for the up-scaling of fish milt cryopreservation for implications in commercial seed production at hatchery. Viveiros *et al.* (2000) reported post-thaw dilution of 1:20, 1:200 and 1:2000 of cryopreserved milt in African catfish *Clarias gariepinus* using 1 ml cryovial. Hatching percentage was comparable with 1:20 (82%) and 1:200 (78%) upon post-thaw dilution of cryomilt which was already diluted 1:10 during freezing. So, possibility of post-thaw dilution, can be a useful strategy to store more genetic material in the small Dewar flask and will improve cost-effectiveness.

Use of premix for cryopreservation

For successful cryopreservation of fish milt, extender is the most important component and need precise weighing to prevent activation of spermatozoa (Scott and Baynes, 1980). Premix of salt compositions for extender preparation is likely to be a convenient and hatchery friendly strategy for saving cost, compensating lack of technical competence and also it saves time for weighing each salt accurately to prepare the extender. This is particularly important in the cases where the cells are to be cryopreserved immediately to avoid quality loss. Our results suggest that when premix is used to prepare extender instantly and sperm cells are cryopreserved and used to fertilise the freshly derived eggs, there was no significant difference in hatching success compared to freshly prepared extender. This approach will have incredible advantage in field condition, which would help to avoid carrying a sensitive weighing balance. Preweighed and premixed salts in vacuum-packed sachets can be supplied to hatchery managers for milt cryopreservation.

The statistical analysis related to hatching percentage of cryopreserved milt stored in straws, cryovials and different dilutions (1:3 and 1:6) reported in the present study has provided possibility of preserving cryomilt in bigger cryovials in 1:3 dilution and on-site dilution with equal volume of extender to fertilise double quantity of eggs. Our observation enables use of less volume of liquid nitrogen to cryopreserve large volume of genetic material. We also observed that the hatching percentage is not significantly affected by the use of larger cryovials. These observations suggest that use of 5 ml cryovial with milt preserved with extender 7 in 1:3 dilutions, upon equal post-thaw dilution can be used to fertilise 67,500 eggs. This approach, as we proved through experimentation, will have significant implications for small-scale farmers for production of genetically diversified carp seed for aquaculture. For a seed producer, it will be lucrative to store more sperm per drop of liquid nitrogen. Furthermore, for cryopreservation of milt at farm site, use of premix of extender salts can be another alternative approach to preserve the valuable genetic materials at field level, without investing on laboratory infrastructure.

The cryopreservation procedures have promise of economic viability, in view of the results aimed to customise the procedures for low-cost and semitechnical carp breeding sector. In a normal farm, with healthy breeders without any constraints, the natural breeding with or without hypophysation can be effective means of seed production. However, cryopreservation of sperm can be an added advantage in specific conditions of asynchrony of two sexes, or low male ratio or where germplasm exchange is necessary to improve broodstock. The technology can assist to overcome the inadequate availability of quality milt especially during early or end of season, when seed prices are higher. In such cases, high volume fertilisation, as tested in this study is important. Future work to develop suitable semiautomatic tools and processing large-quantity of milt for freezing are important research areas to aid commercial use of the technology. In conclusion, we believe that cryopreservation approach can be used as a potential tool in aquaculture to produce genetically diversified seed.

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