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Semen characteristics and optimisation of sperm cryopreservation protocol of a commercially important minor carp *Labeo gonius* (Hamilton, 1822)

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Abstract

This study aimed to develop a suitable protocol for the semen cryopreservation of a commercially important minor carp *Labeo gonius*, to aid in seed production and *ex-situ* conservation efforts. Semen characteristics were analysed, revealing a pH ranging between 7.4-7.7, an average osmolality of 276±15 mOsmol kg^{-1} and sperm counts ranging from 25.4×10^9 to 34.2×10^9 cells ml^{-1}. Nine extenders were screened and three (8, 9B and 9C) were selected based on their high sperm activation (over 70% with water as activator) and sperm motility time (>40 s) after Principle Component Analysis (PCA). Cryopreservation involved a milt to diluent ratio of 1:6, with 10% DMSO as a cryoprotectant. Diluted milt was loaded into 0.25 ml straws, equilibrated and stored in liquid nitrogen. The selected extenders (8, 9B and 9C) demonstrated hatching rates of 61.1±0.23, 81.3±0.19 and 83.7±2.7%, respectively and survival rates of 44.6±1.2, 45.0±2.64 and 46.7±1.52%, respectively. The control group using fresh sperm had hatching and survival rates of 84.99±0.88 and 48.6±1.52%, respectively. Extenders 9C and 9B, containing 10% DMSO and sugar, showed significantly higher hatching and survival rates than extender 8. However, there were no significant differences in hatching or survival rates between cryopreserved sperm and the control group, except in extender 8. The results clearly indicated that, extenders 8, 9B and 9C supplemented with 10% DMSO can be recommended for semen cryopreservation of *L. gonius*, contributing to *ex-situ* conservation and quality seed production.

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Introduction

Cryopreservation of sperm is one the most effective tools of *ex-situ* conservation (Noor *et al*., 2018). It is a widely accepted technique for the conservation of biological material at ultra low temperatures, usually at -196°C in liquid nitrogen. At this temperature, cells remain genetically viable, with their structure and functionality maintained in a reversibly inactive metabolic state (Pegg, 2007). Cryopreservation of milt has many implications mainly for the conservation of fish species, to produce genetically diversified fish seed (Tiersch and Green, 2011; Tsai and Lin, 2012), reducing the cost of production by reduced maintenance of the male broodstock (Rao *et al*., 1989), evolving desired genotype through cross breeding (Diwan *et al*., 2010), eliminating the problem of asynchronous gamete production (Cabrita *et al*., 2010; Varkonyi *et al*., 2019), availability of milt throughout the year, easy transportation and time-independent distribution of genetic material.

Carps contribute 95% of the total freshwater fish production in Indian aquaculture (FAO, 2014), with the three Indian major carps (IMCs) being the choice of preference for decades. Studies have revealed that the incorporation of minor carps, as a companion crop, in the conventional major carp culture has proved to be an effective strategy to enhance production with high economic return as they fetch 20-30% higher market price as compared to the IMCs (Das and Mishra, 2016). *Labeo*

gonius, a medium carp of cyprinidae family, commonly known as 'Kuria labeo' or 'Gonius' has gained huge popularity among fish farmers and proved to be a potential candidate species for captive breeding and culture (Das *et al*., 2010). It is widely distributed in the major freshwater bodies of West Bengal, Odisha, Uttar Pradesh, Bihar, Rajasthan, Madhya Pradesh, Punjab and in many North-Eastern states of India (CAMP, 1998; Mohanta *et al*., 2008). The species has been widely accepted as cultivable fish in Assam. Its flesh does not contain much bones and provides good flavour making it a preferred fish among consumers (Chonder, 1999). It is highly nutritious and is a rich source of calcium, protein and fatty acids (Afroz and Begum, 2014). Interestingly, the fish is having many medicinal properties and is used to cure obesity in villages of Dibrugarh District in Assam State (Kalita *et al*., 2005). Considering the potentiality as new candidate species in aquaculture as well as the conservation aspect of this fish species, the present study was aimed at the development of a suitable and efficient sperm cryopreservation protocol for *L. gonius*.

Materials and methods

Ethics statement

This study was approved by the Animal Ethics Committee of ICAR-National Bureau of Fish Genetic Resources (ICAR-NBFGR) Lucknow. All the animals used in this study were handled according to the guidelines for animal experimentation.

Experimental site

Experiments were conducted at the sperm cryopreservation laboratory and live fish germplasm resource centre of ICAR-NBFGR, Lucknow, India.

Selection and conditioning of brooders

Brood fishes of *L. gonius* were procured from natural water bodies and maintained in earthen ponds at the live germplasm resource centre facility of ICAR-NBFGR, Lucknow. Fishes were fed, twice a day at 3% of body weight with a commercial carp feed (Growel feeds Pvt. Ltd., India) containing 28% protein. Fully ripen male and female brooders (weight 250-280 g) were selected and conditioned under running water for 48 h to ensure emptiness of the gut.

Hormone inducement, milt collection and quality assessment

Selected male brooders were anaesthetised using 2-Phenoxyethanol (0.4 ml l^{-1}) and were then subjected to hormonal induction using "Ovafish" (Bhoomiaqua International Maharashtra, India) at 0.2 ml kg^{-1} body weight, intraperitoneally. After 4 h of hormonal induction, milters were anesthetised and the milt was collected carefully in dry plastic boxes (50 ml capacity). Separate plastic boxes were used for milt collection from each male, with proper labelling. The milt boxes were immediately placed over crushed ice covered with aluminium foil and milt quality was checked using a compound microscope. To assess the sperm motility, a small amount of fresh milt (1 µl) was placed on a slide, covered with a coverslip and examined the sperm cells using a compound microscope at a magnification of x400. Samples containing motile spermatozoa were discarded. Immotile spermatozoa were stimulated by adding distilled water and only those samples exhibiting visual motility exceeding 70% were chosen for further investigations (Kumar *et al*., 2023).

Quantification of spermatozoa

The concentration of spermatozoa in fresh milt samples was assessed using haemocytometer counts (Pioli, 2019; Kumar *et al*., 2023). A solution of neutral buffered formalin (NBF) at 10% concentration, containing a tinge of Eosin, was utilised to dilute and immobilise the milt before loading. For quantification of spermatozoa, 100 µl of fresh milt was added in a vial containing 400 µl of NBF solution. We employed serial dilution to sufficiently dilute the milt to facilitate counting. The number of cells in each of the four large corner squares were recorded and used the average value to calculate the sperm density using the formula:

Spermatozoa per ml = Total spermatozoa/4 x Dilution factor x 10,000

Estimation of pH and osmolality of seminal plasma

Fresh milt (2 ml) was centrifuged for 10 min at 10000 rpm to separate seminal plasma at 4°C. The collected supernatant was tested for pH with pH meter (INOLAB pH meter) and osmolality with osmometer (KNALIER semi-micro osmometer).

Screening of extenders for milt cryopreservation

The composition of nine extenders previously evaluated for various fish species were tested in *L. gonius* (Table 1) and numbers were assigned for convenient representation. The compositions of different extenders used in the experiments were modified as: Extender 3 (Extender V2e of Stein and Bayrle, 1978); Extender 6

Table 1. Chemical constituents (mg 100 ml^{-1}) of different extenders used for cryopreservation of sperm of *L. gonius*

Extenders → / Chemicals ↓	3	3C	6	7	7A	8	8A	9B	9C
NaCl	750	750	730	750	750	440	440	650	650
KCl	38	38	38	20	20	620	620	142	142
$CaCl_2$	-	-	23	20	20	22	22	30	30
$NaHCO_3$	200	200	750	20	20	20	20	-	-
$MgCl_2$	-	-	-	-	-	08	08	-	-
$MgSO_4$	-	-	23	-	-	-	-	20	08
NaH_2PO_4	-	-	41	-	-	-	-	-	-
Glucose	100	100	-	-	100	-	100	54	54
Fructose			100						
Lacithin			750						
Glycine	-	-	-	-	50	-	50	-	-
Mannitol	-	-	250	-	-	-	-	-	-
Egg yolk	20 ml	-	-	-	-	-	-	-	-

Compositions are based on: Extender 3 = (Extender V2e of Stein and Bayrle, 1978); 6 = (Extender 164 of Ott and Horton, 1971); 7 and 8 = (Extender 1 and 2 of Kurokura, *et al.*,1984); 9C = NBFGR annual report 2020: 7A, 8A and 9B = NBFGR modify Extender, 3C = 3 without egg yolk

Table 2. Selection of extenders for milt cryopreservation of *L. gonius*

Extender	Sperm motility in fresh milt (without activation)	Activation upon adding water (%) (Mean±SD)	Sperm survival time (s) (Mean±SD)	Remarks
3	Nil	46.67±5.78	33±10.4	Not selected
3C	Nil	58.33±2.86	30 ±0.0	Not selected
6	Nil	31.67±7.63	36±7.6	Not selected
7	Nil	68.33±2.88	35±0.0	Not selected
7A	Nil	66.67±2.88	26±2.9	Not selected
8	Nil	76.67±5.77	40±8.7	Selected
8A	Nil	23.33±5.77	25±0.0	Not selected
9B	Nil	73.33±5.77	50.7±2.08	Selected
9C	Nil	81.67±2.88	54.5±1.53	Selected

(Extender 164 of Ott and Horton, 1971); Extenders 7 and 8 (Extender 1 and 2 of Kurokura, *et al*., 1984); Extender 9C (NBFGR, 2020); 7A, 8A and 9B (NBFGR modified extenders) and 3C (extender 3 without egg yolk). A dilution ratio of milt: extender was kept at 1:6 for all the extenders. For screening of extenders, 2 µl fresh milt was diluted in 20 µl extender and 2 µl of the diluted milt sample was checked microscopically. If activated sperms were observed in the diluted milt, the extender was rejected, otherwise proceeded for further screening and further 2 µl diluted milt sample mixed with 20 µl water was observed instantaneously under the microscope. Again, if there was no activation of sperms in the water-diluted milt sample, the extender was rejected and if the activated sperms were observed, the life span of sperms and activation percentage was recorded (Table 2). Similarly, the process was repeated thrice for the screening of each extender including fresh milt treated as control. Based on the Principal Component Analysis (PCA) evaluated over four parameters: osmolality, pH, sperm activation (%), and sperm survival time with a 30x4 data matrix, three extenders (8, 9B and 9C) were selected for the cryopreservation of milt (Fig. 1).

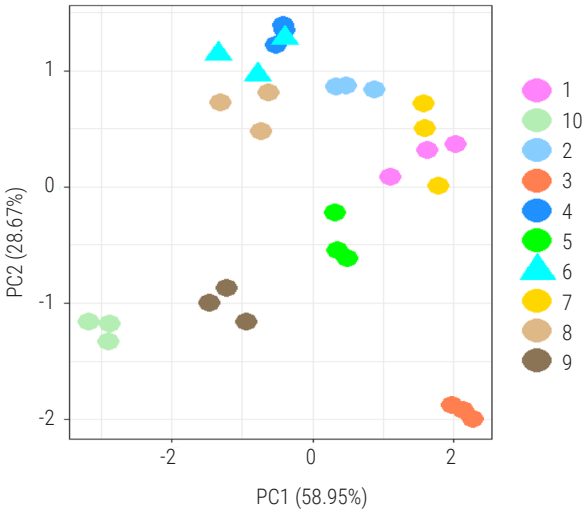

Fig. 1. Bi-variate principal component plot for evaluation of extenders and Control over four variables; Osmolality, pH, Sperm activation (%), Sperm survival time

Cryopreservation of milt

Selected extenders (8, 9B and 9C) were prepared and stored in chilled condition in the icebox. Fresh milt was used as control in the experiment. Dimethyl sulphoxide (DMSO: 10% v/v; HIMEDIA Laboratories Pvt. Ltd, Mumbai) was used as a cryoprotectant. After quality assessment, non-activated milt samples were pooled, diluted with diluent (extender and cryoprotectant) in the ratio 1:6 (milt: diluent) and filled in 0.25 ml straws (Bal Krishna Plastikraft, Delhi, India). Straws were filled with manual filling system by sucking from the cotton-plugged side and sealed with PVA (Polyvinyl alcohol) powder.

The straws were then equilibrated in the cold chamber (4°C cooling chamber) for 10 min including the filling time. After equilibration, straws were exposed to liquid nitrogen (LN2) vapor phase on a freezing stand at 2.5 cm above surface of LN2 in Styrofoam box to attain a temperature of -120°C to -150°C for 10 min and then plunged into liquid nitrogen (-196°C). The technique was standardised using a programmed freezer that cools at a rate of -10°C per min. The straws were then placed in labelled canisters and stored in cryo-cans for future use.

Fertility trial

Fully ripen female and male brooders after conditioning for 48 h were used for fertility trial. Three male and three female brooders were anaesthetised and injected *via* intraperitoneal injection of "Ovafish" at 0.2 ml (male) and 0.5 ml (female) per kg body weight, respectively. After 6 h of hormonal induction, milters were anaesthetised and the milt was collected in dry plastic box carefully, ensuring zero contact with any extraneous source of fluids. After checking the motility of the milt from individual fishes, the milt was pooled. Similarly, the eggs from females were retrieved in an airtight plastic box. The experiment was designed as Randomised Block Design (RBD) with three replicates. Approximately, 300 eggs were kept in each replicate. In control group, eggs were fertilised with 36 µl of fresh pooled milt sample. Likewise, eggs were fertilised with one straw of cryopreserved milt of respective treatment groups. Sperm-to-egg ratio were kept at $3x10^6$ sperms per egg. For fertilisation of 8, 9B and 9C group, the straws stored in liquid nitrogen were thawed in water bath at 40°C temperature for 10 s. Straws were cut at both ends; cryopreserved milt was mixed with eggs and activated using 100 µl water. This process was accomplished within 30 s excluding thawing time to ensure proper fertilisation. Fertilised eggs were washed gently 3-4 times with tap water and incubated in flow-through hatchery with water flowrate of 2-3 l per minute (l min^{-1}). Water was replaced at regular intervals of 3 h. Hatching occurred between 60 to 68 h following fertilisation. The hatching percentage

was calculated by counting the number of hatchlings from the total number of eggs in the incubation bowl using the formula:

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

Survival and growth of fry

A total of 200 individuals per treatment were maintained in a flow-through system. Fry with an average initial length of 0.44±0.38 mm and an average initial weight of 1.43±0.12 mg were reared for 60 days in flow-through hatchery with a water flow rate of 3-4 l min^{-1}. Survival and different growth parameters were recorded at day 60. The following formulae were used to calculate different parameters.

Length gain of larvae (cm) = Average final length of larvae - Average initial length of larvae

Weight gain of larvae (mg) = Average final weight of larvae - Average initial weight of larvae.

Percent gain in length = [(Average final length of larvae - Average initial length of larvae)/ Average initial length] × 100

Percent gain in weight = [(Average final weight - Average initial weight) /Average initial weight] × 100

Specific growth rate (SGR) = [(lnW2 - lnW1)/ (T2 - T1)] × 100

where, W2 = Final live body weight (g) at time T2, W1 = Initial live body weight (g) at time T1

Survival rate = (No. of larvae alive at the end/Total No. stocked) ×100

Statistical analysis

Experimental data were analysed statistically using SPSS ver. 16 and Adegenet R. Hatching percentage was subjected to Arcsine transformation before analysing for one-way ANOVA. Growth parameters were analysed with one-way ANOVA after the log transformation of data. Classical one-way analysis of variance was applied and means were compared with Tukey's *post-hoc* test. All the recorded parameters were also combined and subjected to multivariate analysis; Principal component analysis (PCA) and Discriminant Analysis on Principal Components (DAPC). The level of statistical significance was set at 0.05.

Results

Quality estimation of milt

Fresh milt was analysed for sperm count, pH of seminal plasma, the osmolality of seminal plasma, sperm motility percentage and life span of sperms after water activation. The number of sperms in different milt samples ranged from 25.4×10^9 to 34.2×10^9 cells ml^{-1}. The pH of the seminal plasma ranged between 7.4-7.7 and the milt had an average osmolality of 276±15 mOsmol kg^{-1}. In fresh milt samples, the motility percentage of sperms ranged from 25 to 80% in different extenders with a life span of 25 to 50 s. During the motility test, the sperm displayed active forward and passive Brownian movements.

Fertility trial

Sperm to egg ratio of $3x10^6$ sperms per egg was used in the fertility trial when $1x10^9$ spermatozoa were used for 300 eggs. Eggs were fertilised with cryopreserved milt using different extenders and fresh milt was used as control. The hatching rate was recorded and is presented in Table 3. No significant difference in hatching rate was observed between control, 9C and 9B whereas it was significantly low in extender 8 ($p<0.05$).

Growth performance

Growth performance and survival of fish larvae are presented in Table 4. The recorded initial average length and initial average weight were 4.30±0.07 mm and 1.43± 0.12 mg, respectively for all the treatments. At the end of the experiment, the final average length (mm) and the final average weight (mg) of the larvae in control and different treatments were 11±2.0, 13.3±1.15, 11.67±1.15 and 12.67±1.52 and 62±2.64, 65.6±4.05, 66.6±2.51 and 67±4.0, in extenders 8, 9B and 9C respectively and no significant difference was observed among the treatment groups ($p>0.05$). No significant difference was seen in survival also.

Multivariate analysis

After selecting the three extenders by virtue of PCA, further analysis, to observe the performance-based differences among significant extenders and control over targeted variables on scores over discriminant functions reveals the control and different extenders as distinct centroids connected with a minimum spanning tree reflecting mean-based performance differences (Fig. 2). Evaluation of the performance of four groups, consisting of a control and three extenders, over a discriminant function yielded a score-density that reflected nearly similar performances, as illustrated by the mixing graph in Fig. 3.

Discussion

The present study shows that the motility of spermatozoa varies with different extenders. Three extenders (9C, 9B and 8) having sperm activation percentage greater than 70% and sperm life span (swimming duration) of more than 40 s were used for cryopreservation. The percentage survivability of sperms after water activation greater than 70% is best for cryopreservation (Coser *et al*., 1984; Piironen, 1993; Lahnsteiner *et al*., 1997; Routray *et al*., 2006). Survival time of sperms is also an important parameter in the assessment of milt quality. Mean survival duration of sperms was recorded maximum in 9C (54.5±1.53 s) followed by 9B (50.1±2.08 s) and 8 (40±8.7 s). Survival duration in extender 9C and extender 9B was not significantly different ($p>0.05$) where both were significantly higher ($p<0.05$) than that of extender 8. This was possibly due to

Table 3. Hatching rates in different treatment groups

Treatment*	Hatching (%) (Mean±SD)
Control	84.99±0.88 [a]
9B	81.3±0.19 [a]
8	61.1±0.23 [b]
9C	83.7±2.7 [a]

*Each treatment comprised three replicates.

Table 4. Growth performance of *L. gonius* larvae of different treatment groups

Parameters \ Treatments	Control	8	9B	9C
Initial average length (mm)	4.3±0.07 [a]	4.3±0.07 [a]	4.3±0.07 [a]	4.3±0.07 [a]
Final length (mm)	11 ± 2.0 [a]	13.3±1.15 [a]	11.67±1.15 [b]	12.67±1.52 [a]
Initial average weight (mg)	1.43 ± 0.12 [a]	1.43 ± 0.12 [a]	1.43 ± 0.12 [a]	1.43 ± 0.12 [a]
Final weight (mg)	62± 2.64 [a]	65.6 ± 4.05 [a]	66.6 ± 2.51 [a]	67 ± 4.0 [a]
Length gain of larvae (mm)	6.7± 1.7 [a]	9.0± 1.2 [b]	7.4± 1.2 [c]	8.4± 1.5 [a]
Weight gain of larvae (mg)	60.57± 2.64 [a]	64.23± 4.04 [a]	65± 2.51 [a]	60± 5.64 [a]
Percent gain in length	155±40.28 [a]	210.07± 26.8 [a]	171.3± 26.85 [a]	194.5± 35.4 [a]
Percent gain in weight	4235.6± 185 [a]	4492.07± 282.6 [a]	4562.6± 175.9 [a]	4258.9± 385.6 [a]
Specific growth rate (SGR)	4.100±0.044 [a]	4.159±0.0642 [a]	4.175±0.038 [a]	4.10±0.093 [a]
Survival (%)	48.6 ± 1.52 [a]	44.6± 2.08 [a]	45.0 ±2.64 [a]	46.7± 1.52 [a]

Value in the same row with different superscripts are significantly different ($p < 0.05$).

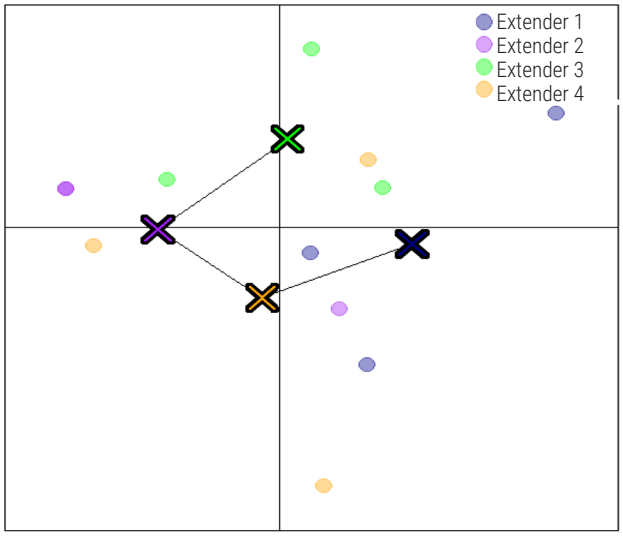

Fig. 2. Bi-variate Discriminant function plot for evaluation of control and selected Extenders on hatching % and growth variables

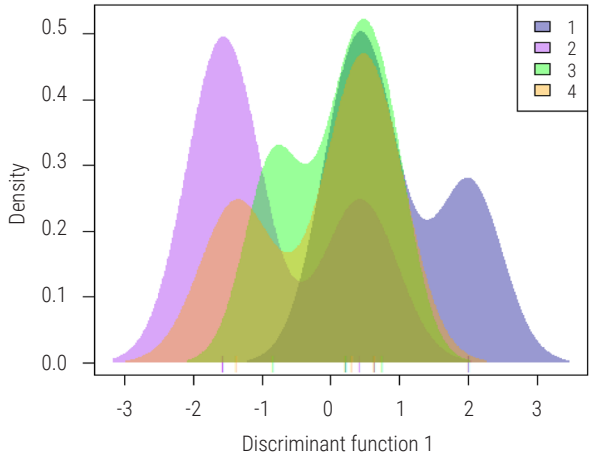

Fig. 3. Performances of control and selected extenders on different variables over discriminant function 1

the presence of sugar in extender 9C and extender 9B. Presence of sugars is often attributable to successful cryopreservation (Warnecke and Pluta, 2003). Similar results were observed by Warnecke and Pluta (2003) and Lahnsteiner *et al*. (1997) where they found that addition of sucrose improves the effectiveness of the extender. Sugar significantly improved post-thaw motility duration and viability rates of sperms (Bozkurt *et al*., 2016).

Fish sperm motility is influenced by osmolality of seminal fluid (Morisawa, 1985) and fish sperms are immotile in the range of 270-300 mOsmol kg^{-1} (Alavi and Cosson 2005; Yang *et al*., 2007). In the present study, the mean osmolality recorded was 276±15 mOsmol kg^{-1} which is comparable to 269±5 mOsmol kg^{-1} reported by Verma *et al*. (2009) and 263±6 mOsmol kg^{-1} oberved by Sahu *et al*. (2011) in *L. rohita*. In the present study, the seminal plasma was found slightly alkaline (7.6 ±0.19). Verma *et al.* (2009) reported that the pH of the seminal plasma of *Labeo rohita, Catla catla* and *Cirrhinus mrigala* is in the range of 7.3-7.9. The standard method for determining sperm density using a haeomocytometer, is generally used for spermatozoa count (Buyukhaitoppoglu and Holtz, 1984). In our study, the mean milt cell count of *L. gonius* was $32\pm0.65 \times 10^9$ cells ml^{-1}. Gupta and Rath (1993) observed that sperm cell counts ranged between 2.1 and 2.5×10^7, 3.0 and 3.25×10^7 and 2.0 and 2.5×10^7 cells ml^{-1} for catla, rohu and mrigal, respectively. The reported milt cell count in common carp was $48.75\pm0.9 \times 10^9$ ml^{-1} (Sharma *et al*., 2017) and $8.55\pm2.77 \times 10^9$ ml^{-1} in rohu (Khan *et al*., 2015) and 5.9×10^9 ml^{-1} to 6.5×10^{10} ml^{-1} in *Labeo bata* (Noor *et al*., 2018). Broodstock feed regime, age and breeding season attribute to the difference in sperm quality, including sperm count (Tekin *et al*., 2003). Cryoprotectants are added along with extenders to protect sperm cells from stress during cooling and freezing. They help prevent cold shock and minimise cryo-injury by reducing external ice crystal formation (Kurokura *et al*., 1984; Ponniah *et al*., 1998). DMSO (10%) was used as cryoprotectant in the present study which gave satisfactory results. Several authors reported that compared to methanol, DMA and glycerol, DMSO performs better in cyprinids (Withler 1980; Durbin *et al*., 1982; Kumar, 1988; Gupta and Rath, 1993; Gupta *et al*., 1995). DMSO has a higher efflux capacity and a lesser temperature-dependent process of cellular inflow making it one of the best and most preferred cryoprotectant (Huang *et al*., 2018). Earlier studies of Withler and Lim (1982); Lakra and Krishna (1997) found that 10% DMSO has excellent permeation capacity,

low toxicity, fast rate of penetration and interaction with sperm membrane phospholipids in freshwater fish species. DMSO at 10% (v/v) concentration of the diluents was found effective for Indian major carps (Routray *et al*., 2006), *Labeo calbasu* (Nahiduzzaman *et al*., 2011), *Labeo rohita* (Ahammad *et al*., 2003) and *Labeo bata* (Noor *et al*., 2018).

In the fertility trial conducted using cryopreserved milt preserved with extenders 9C, 9B and 8, along with fresh milt as control, no significant difference in hatching rate was observed between the control, extender 9C and extender 9B, whereas it was significantly low ($p<0.05$) in extender 8. No previous records are available for *L. gonius* in terms of hatching using cryopreserved milt. However, Nahiduzzaman *et al.* (2011) observed 37% hatching in *Puntius sarana* eggs fertilised with cryopreserved milt in Alsever's solution with 10% DMSO, while Noor *et al.* (2018) observed hatching rate of 18.4±6.8% in *Labeo bata* eggs fertilised with the same mix of diluents. Vuthinphandchai *et al*. (2015) reported a 45.4±51.2% hatching rate in silver barb (*Barbodes gonionotus*) using calcium free Hank's balanced salt solution (CF-HBSS) with DMSO. Sharma *et al*. (2017) reported a hatching rate of 63-69% in amur carp (*Cyprinus carpio haematopterus*).

Comparison of growth performance of offsprings generated from cryopreserved semen samples with that of the control group, showed no significant difference in the survival rate after 60 days of rearing. No previous records are available on the growth performance of *L. gonius* produced through cryopreservation, although a study related to the polyculture of Kuria labeo (*L. gonius*) with major carps found that *L. gonius* is compatible with other major carps (Jena and Das, 2011; Jena *et al*., 2011). Although they observed that the growth performance of Kuria labeo was inferior to that of mrigal, the better compatibility of Kuria labeo with rohu helped this combination yield a biomass equivalent to the mrigal-rohu combination, suggesting the feasibility of using *L. gonius* as an alternative species to mrigal in the major carp polyculture system without compromising the total biomass yield.

Extenders 8, 9B and 9C yielded encouraging results in these preliminary attempts to develop a cryopreservation protocol for *L. gonius* sperm, with no significant difference in hatching rate compared to the control, except for extender 8. The present study not only contributes to the conservation of *L. gonius* but also provides a useful protocol for sperm cryopreservation . Future research can further refine this protocol for mass-scale semen cryopreservation of this species, facilitating its implementation at the farmers' level.

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