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Short Communication

Effect of Sperm-Egg Ratio and Thawing Protocol on Hatching Success of Cryopreserved Rohu (*Labeo rohita*) Milt

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GS: Conceived and conducted the experiment, carried out broodstock management, milt cryopreservation, fertilization trials, data collection and statistical analysis, and drafted the initial manuscript. SK: Designed the research framework, supervised the study, guided interpretation of fertilization and hatching results, and provided technical inputs. DK: Assisted in experimental planning, validated statistical analysis, reviewed sperm–egg ratio optimization strategy, and critically revised the manuscript. OW: Contributed to methodology, monitored trials, and reviewed the results section. IH: Assisted in literature review, supported data interpretation, provided constructive suggestions, and contributed to manuscript editing and final approval.

Key words
Cryopreservation, *Labeo rohita*, Sperm–egg ratio, Thawing temperature, Hatching success

ABSTRACT

Cryopreservation of fish milt is an effective strategy to ensure year-round availability of quality gametes for aquaculture and conservation programs. However, fertilization efficiency using cryopreserved sperm is strongly influenced by sperm–egg ratio and thawing protocol, particularly when large-volume cryovials are used for hatchery-scale application. The present study evaluated the effect of different sperm–egg ratios and thawing temperatures on the hatching success of cryopreserved rohu (*Labeo rohita*) milt. Milt collected from hormonally induced males was cryopreserved using selected extender and cryoprotectant combinations and stored in 2 ml, 5 ml, and 12 ml cryovials. Frozen samples were thawed at standardized temperatures specific to each vial size and used for fertilization trials with eggs obtained from induced females. Fertilization was carried out using varying volumes of cryopreserved milt, and hatching percentage was recorded under standard hatchery conditions. Hatching success varied significantly among treatments, with the highest hatching rate (51%) obtained using one spoonful of eggs fertilized with one 2 ml vial of cryopreserved milt. Among different vial sizes, optimal hatching was achieved at a thawing temperature of 40°C for 2 ml and 5 ml cryovials, while 12 ml cryovials showed maximum hatching (47.9%) at 45°C. Fresh milt produced significantly higher hatching (79%) compared to cryopreserved treatments. The results demonstrate that appropriate optimization of sperm–egg ratio and thawing protocol enables effective use of large-volume cryovials, supporting the feasibility of upscaling rohu milt cryopreservation for commercial seed production.

One of the most significant freshwater species is the rohu (*Labeo rohita*), which is a member of the Cyprinidae family. In essence, it is a column feeder and an omnivore (Azim *et al*., 2001). Rohu is a promising species for farming due to its rapid growth rate, high market value, and rising customer demand. According to Dewan *et al*. (1977) it is widely practiced in China, Vietnam, Indonesia, Thailand, the Philippines, India, Bangladesh, and Pakistan.

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Ensuring consistent seed availability is a major challenge in carp aquaculture, particularly due to seasonal breeding, variability in broodstock quality, and environmental constraints. Cryopreservation is an effective method for the long-term storage of viable sperm and can provide a year-round supply of male gametes. The technique is useful for the cryopreservation of sperm from genetically superior males for later use, in the transportation of sperm, and for use in breeding programs such as cross breeding, genetic studies or conservation of endangered species. The success of artificial fertilization using cryopreserved sperm depends on several critical factors, sperm quality, extender and cryoprotectant combination, thawing protocol and sperm-egg ratio. Usually, the factors that can influence storage of fish milt are considered. For example, the quality of the broodstock (i.e. age, breeding season, diet, health and management), fresh milt quality and

quantity (i.e. volume, sperm concentration and motility), and the conditions of milt storage (i.e., temperature, artificial seminal plasma, time, volume, perturbations and atmosphere) (Contreras *et al*., 2020). While small-volume straws and cryovials are commonly used, they limit the quantity of sperm stored per unit of liquid nitrogen. Upscaling cryopreservation using larger cryovials could improve operational efficiency in hatcheries, provided that thawing protocols are optimized to maintain sperm viability. Therefore, the present study aimed to evaluate the effect of different sperm–egg ratios and thawing protocols on hatching success of cryopreserved rohu (*Labeo rohita)* milt, with special emphasis on the feasibility of large-volume cryovials for hatchery-scale application.

Materials and methods

Broodstock selection

Sexually mature male and female rohu broodstock were selected based on external morphological characteristics during the breeding season. The male broodfish were held separately in shadowed tanks and supplied with well aerated water. In accordance with carp cultivation protocol, tanks were prepared and managed. A commercial diet was given to broodstocks at a rate of 2% body weight per day until they were satiated.

Broodstock conditioning

The brood stocks were collected from ponds 12-24 h prior to planned stripping and transported to the hatchery for acclimatization. Male brood fishes were conditioned in a 5000-liter tank under continuous showering condition for 6 h before hormonal induction.

Milt collection

The male broodfish were induced with WOVA-FH (Biostadt India Limited, Mumbai) at a dose of 0.2 mg/kg body weight. Prior to the collection of milt, the male brood fish were anesthetized using phenoxyethanol (Bernath *et al*., 2016) at 0.4 ml/L. The abdomen was wiped with tissue paper to remove moisture and water if any, to avoid unexpected activation of the spermatozoa. The milt was collected 4 h after hormonal administration by gentle hand stripping into dry plastic sperm collection box.

Cryopreservation of milt

Selected extender and cryoprotectant combinations were used for cryopreservation. Milt was diluted at a ratio of 1:5.3:0.7 (milt: extender: cryoprotectant). Extender was first mixed with milt, followed by the gradual addition of cryoprotectant. The diluted milt was loaded into 2 ml, 5 ml, and 12ml cryovials using stepper (NICHIRYO, F 19500031, Japan) and equilibrated for 12 min before vapour-phase freezing. Cryovials were placed on a steel stand positioned above the liquid nitrogen surface during vapour-phase freezing. The distance from the liquid nitrogen surface was 2.5 cm for 2 ml cryovials, 2.0 cm for 5 ml cryovials, and 1.5 cm for 12 ml cryovials. Samples were exposed to nitrogen vapour for 12 min, achieving an approximate cooling rate of −10 °C min^{-1} before plunging into liquid nitrogen (−196 °C) for storage.

Thawing protocol

Cryovials were removed from liquid nitrogen and immediately subjected to thawing in water bath (REMI, RWB 6, India). Cryovials were thawed in an ice–water mixture (10:90, ice: water, v/v) until complete melting of the contents. Subsequently, samples were exposed to the designated thawing temperatures (40–55°C) according to the experimental treatments before fertilization.

Fertilization and sperm-egg ratio

Female broodfish were induced with WOVA-FH at a dose of 0.5 mg kg^{-1} body weight via intraperitoneal injection. Eggs were collected by hand stripping 6 h after hormonal induction following anesthesia with phenoxyethanol. Eggs from a single female were pooled and divided into treatment batches in triplicate.

For fertilization trials, one spoonful of eggs (approximately 16,000 eggs) was fertilized with varying volumes of cryopreserved milt according to treatment. Thawed milt was mixed thoroughly with eggs, and spermatozoa were activated by adding 5 ml of tap water. For control treatments, 300 µl of fresh milt collected 4 h after hormonal induction was used. Fertilized eggs were incubated under standard hatchery conditions, and hatching percentage was recorded.

Statistical analysis

All experiments were conducted in triplicate. Data were expressed as mean±standard deviation (SD). Statistical differences among treatments were analyzed using one-way ANOVA followed by post hoc multiple comparison tests. Differences were considered significant at $p < 0.05$.

Results

Effect of sperm-egg ratio on hatching

Hatching success varied significantly among different sperm–egg ratios (Table I). Based on sperm concentration analysis and the volume of milt used for fertilization, the optimal treatment corresponded to approximately 1.88×10^6 spermatozoa per egg. The treatment using one spoonful of eggs fertilized with one vial (2 ml) of cryopreserved

milt resulted in the highest hatching rate (51%), followed by one spoon of egg with two vials (25%). Lower hatching rates were observed in treatments using one spoonful of eggs with 1.5 vials (22.91%) and 1.5 spoonful of eggs with one vial (21.94%). The control group fertilized with fresh milt exhibited the highest hatching rate (79%).

Table I. Effect of sperm-egg ratio on hatching percentage in cryopreserved rohu milt.

Sperm egg ratio	Hatching (%)
1 spoon 1 vial	51±9.0^{b}
1 spoon 1.5 vial	22±6.0^{c}
1.5 spoon 1 vial	21±6.0^{c}
1 spoon 2 vial	25±1.3^{c}
Control	79±2.0^{a}

Values with different superscripts differ significantly ($p < 0.05$).

Effect of cryovials size and thawing temperature

Thawing temperature significantly affected hatching success across different cryovial sizes (Table II). The 2 ml and 5 ml cryovials thawed at 40°C yielded hatching rates of 51% and 48%, respectively. For 12 ml cryovials, the highest hatching rate (47.9%) was obtained at 45°C, while higher thawing temperatures of 50°C and 55°C resulted in reduced hatching rates (45.3% and 40.82%, respectively). The control group, using fresh sperm, had the best hatching rate (79%).

Table II. Effect of vial size and thawing temperature on hatching percentage in cryopreserved rohu milt.

Size of vial	Thawing temperature	Hatching percentage
Control	40° C	79%
2 ml	40° C	51%
5 ml	40° C	48%
12 ml	45° C	47.9%
	50° C	45.3%
	55° C	40.82%

Discussion

Hatching success obtained in the present study is comparable with earlier reports on cryopreserved carp sperm. Cejko *et al*. (2022) reported hatching rates ranging from 36% to 48%, depending on the type of extender and sperm-to-egg ratio. Kumar *et al*. (2023) reported the use of approximately 1.5×10^{6} sperm cells per egg for fertilization using cryopreserved milt in common carp. It is important to observe that comparing the hatching % without comparing the sperm: Egg ratio will have a major effect on the results (Di Lorio *et al*., 2019). Previous studies have reported wide variation in optimal sperm–egg ratios among species, including 8×10^{5} sperm per egg in silver barb (Vuthiphandchai *et al*., 2015), 4.6×10^{6} sperm per egg in *Brycon orbignyanus* (Maria *et al*., 2006), 1×10^{5} sperm per egg in common carp (Warnecke and Pluta, 2003), and 5×10^{5} sperm per egg in rainbow trout (Sahin *et al*., 2013). The optimal sperm–egg ratio determined in this study (1.88×10^{6} spermatozoa per egg) was higher than values reported for several freshwater species. The relatively higher sperm requirement may be attributed to the use of cryopreserved milt and the reduced egg quality associated with late breeding season conditions.

Previous reports indicate that larger cryovials can be effectively used for fish sperm cryopreservation when appropriate thawing protocols are applied. Kumar *et al*. (2023) reported the hatching rate of using 2 ml and 5 ml cryovials 55.6±11.2 and 53.4±7.6 in common carp. Varkonyi *et al*. (2019) reported higher VSL (Grayling: 45 ± 3 µm/s, Pike: 38 ± 4 µm/s) was observed by the grayling extender using the 10 ml cryovial than with the pike extender in common carp. Beirao *et al*. (2011) reported the hatching rate of 34.5±19.3% in sea bream. Ji *et al*. (2004) reported the use of 1.8 ml cryovial in sea perch with fertilization and hatching rate of 84.8% and 70.1%, respectively. In the present study best hatching rate of using 12 ml cryovial is 47.9% demonstrating the feasibility of large-volume cryopreservation. Through this research, it will help the seed producer to store more sperm per each drop of liquid nitrogen. Further studies required to develop the suitable sperm egg ratio and freezing of fish sperm for large quantity for commercial seed production. Rapid thawing at 40–45°C likely minimized ice recrystallization damage, while higher temperatures (50–55°C) may have caused thermal stress to sperm membranes. The higher sperm requirement observed for cryopreserved milt may be due to reduced post-thaw motility and membrane integrity.

Conclusion

The study demonstrated that hatching success of cryopreserved rohu (*Labeo rohita*) milt is significantly influenced by sperm–egg ratio and thawing protocol. Optimal hatching was achieved when one spoonful of eggs was fertilized with one 2 ml vial of cryopreserved milt, while thawing at 40°C for 2 ml and 5 ml cryovials and 45°C for 12 ml cryovials yielded the best results. Although fresh milt produced higher hatching rates, optimized cryopreserved milt showed satisfactory performance. These findings confirm the feasibility of using large-volume cryovials for upscaled milt cryopreservation and highlight their potential application in commercial carp seed production.

DECLARATIONS

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The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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