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Sperm cryopreservation of the Indian major carp, *Labeo calbasu*: Effects of cryoprotectants, cooling rates and thawing rates on egg fertilization

Md. Nahiduzzaman[a], Md. Mahbubul Hassan[b], Pankoz Kumar Roy[a], Md. Akhtar Hossain[c], Mostafa Ali Reza Hossain[a,*], Terrence R. Tiersch[d]

[a] *Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*
[b] *Department of Fisheries Biology and Genetics, Hajee Mohammad Danesh Science and Technology University, Dinajpur 5200, Bangladesh*
[c] *Department of Fisheries, University of Rajshahi, Rajshahi 6205, Bangladesh*
[d] *Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA*

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ABSTRACT

A sperm cryopreservation protocol for the Indian major carp, *Labeo calbasu*, was developed for long-term preservation and artificial fertilization. Milt collected from mature male fish were placed in Alsever's solution (296 mOsmol kg^{-1}) to immobilize the sperm. Cryoprotectant toxicity was evaluated by motility assessment with dimethyl sulfoxide (DMSO) and methanol at 5, 10 and 15% concentrations. DMSO was more toxic at higher concentrations than methanol, and consequently 15% DMSO was excluded from further study. A one-step cooling protocol (from 5 to 80 °C) with two cooling rates (5 and 10 °C/min) was carried out in a computer-controlled freezer (FREEZE CONTROL® CL-3300; Australia). Based on post-thaw motility, the 10 °C/min cooling rate with either 10% DMSO or 10% methanol yielded significantly higher ($P = 0.011$) post-thaw motility than the other rate and cryoprotectant concentrations. Sperm thawed at 40 °C for 15 s and fresh sperm were used to fertilize freshly collected *L. calbasu* eggs and significant differences were observed ($P = 0.001$) in percent fertilization between cryopreserved and fresh sperm as well as among different sperm-to-egg ratios ($P = 0.001$). The highest fertilization and hatching rates were observed for thawed sperm at a sperm-to-egg ratio of 4.1×10^5:1. The cryopreservation protocol developed can facilitate hatchery operations and long-term conservation of genetic resources of *L. calbasu*.

1. Introduction

Cryopreservation includes the storage of genetic materials at the temperature of liquid nitrogen (−196 °C) without major change in biological integrity potentially for hundreds or thousands of years. Compared to cattle semen, fish sperm cryopreservation is a developing science with only a rudimentary theoretical framework and often empirical advances made by trial and error. Sperm cryopreservation protocols have been developed for more than 200 finfish and shellfish species around the world (Tiersch et al., 2007). In Bangladesh, research on fish sperm cryopreservation started in 2004 (Hossain et al., 2011), and only 15 species have been studied to date. With respect to the degraded biodiversity of Bangladesh, fish sperm cryopreservation could be employed as an effective strategy to save imperiled species by facilitating the storage of gametes in gene banks (Nahiduzzaman et al., 2011).

Labeo calbasu (Hamilton, 1822), an Indian major carp of the family Cyprinidae, which attains a maximum length of 90 cm (Menon, 1999), is a popular food fish and has

* Corresponding author. Tel.: +880 1711 045364; fax: +880 91 61510.
E-mail addresses: zaman_nahid@yahoo.com (Md. Nahiduzzaman), mmhassan46@yahoo.com (Md.M. Hassan), pankoz_22@yahoo.com (P.K. Roy), mahfaa@yahoo.com (Md.A. Hossain), marhossain@yahoo.com (M.A.R. Hossain), TTiersch@agcenter.lsu.edu (T.R. Tiersch).

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potential for commercial aquaculture in Bangladesh and other South Asian countries. The fish has been reported from open waters of Bangladesh and neighboring countries including India, Pakistan, Myanmar, Thailand, and China (Talwar and Jhingran, 1991). In the past, the fish was abundantly available in all of the major rivers and floodplains of Bangladesh and currently, has been listed as an endangered fish (IUCN-Bangladesh, 2000). Development of cryopreservation protocols for this species could be an appropriate tool for ensuring genetic diversity in selective breeding programs and laboratory conservation of sperm for gene banking. A repository of frozen sperm would ensure the accessibility of a large and diverse spectrum of samples with the practical advantages of reducing dependence on synchronous maturation between sexes, facilitation of gamete transportation, and reduction of the maintenance of live broodstock (Melo and Godinho, 2006).

After collection, freshwater fish sperm must be diluted in an isotonic extender solution, which prolongs cell viability by immobilizing the sperm, and prevents death from desiccation and hypoxia (Riley et al., 2004). To develop sperm cryopreservation protocols for any species, immobilization solutions, sperm dilution ratios, activation media, cryoprotective agents, equilibration time, cooling rates, thawing rates, ionic composition and osmolality of samples should be the primary consideration (Tiersch, 2011).

Fish sperm are typically immotile in seminal plasma (Alavi et al., 2009) and therefore, diluents should be prepared with osmolality similar to that of seminal plasma. Among the different cryoprotectants, dimethyl sulfoxide (DMSO) and methanol (which quickly permeates cells) have been tested at different concentrations to establish the equilibration time necessary to protect cells from toxicity, dehydration and ice crystal formation. After equilibration, sperm samples are cooled at a range of rates (often 5–15 °C/min) in the vapor of liquid nitrogen and stored in liquid nitrogen dewars (Cabrita et al., 2005).

Sperm cryopreservation of cyprinid fish has been studied for a considerable number of species around the world and, it is possible to gain a general understanding of the broad range of conditions necessary for cryopreservation. Compared to the number of protocols developed for sperm cryopreservation of the cyprinids, very few studies have been conducted to date for the uniform cryopreservation technique. Although uniform protocol might be useful, there are chances of variation of the spermatological properties, toxicity tolerance level to cryoprotectants that can affect fertilization of eggs with cryopreserved sperm for each of the species. The study reports on the preliminary aspects of sperm cryopreservation of *L. calbasu* therefore, the basic aspects have been addressed rather considering to develop a uniform protocol. The suitability of protocols can be tested by evaluating motility of the thawed sperm. However, the ultimate criterion is the ability to fertilize eggs, and consequently good survival and growth of hatchlings (Honeyfield and Krise, 2000). Furthermore, the sperm-to-egg ratio of cryopreserved sperm needs to be optimized, otherwise, excess sperm could be used for fertilization. Accordingly, the objectives of the present research were to test the following: (1) equilibration of *L. calbasu* sperm with different cryoprotectants in graded concentrations and time; (2) different cooling and thawing temperatures with appropriate cooling and thawing rates, and (3) fertilization capacity and appropriate sperm-to-egg ratios for thawed sperm.

2. Materials and methods

2.1. Collection and rearing of broods

Broods of *L. calbasu* were collected in 2007 and 2008 from two major rivers in Bangladesh – the Jamuna (Bogra District) and the Halda (Chittagong District) and a *haor* (Netrokona District). Collected broods were transported to the rearing ponds of the Fisheries Field Laboratory Complex, Bangladesh Agricultural University (BAU), Mymensingh. In the rearing ponds, the broods were fed commercial diet (35% protein; Paragon Feeds Limited, Bangladesh) twice daily at 4–5% of the body weight of the fish.

2.2. Milt collection, dilution and activation

Milt samples were collected from *L. calbasu* during the breeding season of two successive years (May–July 2008 and 2009). Males were injected intraperitoneally with carp pituitary supernatant (2 mg kg^{-1} body weight) for induction of spermiation. Six hours after injection, gentle pressure was applied to the abdomen and milt samples were collected in plastic tubes containing 1.0 ml Alsever's solution (0.7% NaCl, 0.8% sodium citrate dissolved in 100 ml water; osmolality 296 mOsmol kg^{-1}) which had sufficient osmotic pressure to immobilize the sperm. The collected samples were placed on ice (4 °C) and brought to the Laboratory of Fish Biodiversity and Conservation for further study. Samples were diluted at 1:9 ratio based on results of prior experiments (Hassan et al., 2012) on dilution ratios of *L. calbasu* sperm. The suitability of diluents and dilution ratios were evaluated with respect to percent motility of the stored sperm after activation.

The percent motility of sperm was estimated by visual observations of two observers with three replications for each sample using a light microscope (Novex K-range, Netherland) at 400× magnification. Sperm motility was estimated by adding 10 μl of 0.3% NaCl (96 mOsmol kg^{-1}) as activating medium to 1 μl of fresh milt on a glass slide. The motility was observed within 4–6 s after activation and was expressed as the percentage of sperm that had forward movement.

2.3. Evaluation of sperm concentration

Sperm counts were performed in triplicate by haemacytometer and expressed as the number of cells per ml. Sperm was diluted 4000-fold in 0.3% NaCl, and a droplet of the diluted sperm was placed within a haemacytometer (area of the smallest square = 1/400 mm^2, depth 0.1 mm) with cover slip (Marienfeld, Germany). A total of ten minutes were allowed to sediment all the sperm. The number of spermatozoa in five large squares (area 1/25 mm^2) of the counting chamber was counted at 400× magnification.

2.4. Evaluation of cryoprotectant toxicity

Cryoprotectant concentrations were prepared at 5%, 10%, and 15% in the final suspension (diluent, sperm and cryoprotectant) with five replicates. Either DMSO or methanol was added with Alsever's solution and mixed with fresh spermatozoa to yield the final cryoprotectant concentration in the suspension. The dilution ratio of sperm to Alsever's solution was 1:9, and after preparation the suspension was placed in ice (4 °C). The toxicity of the cryoprotectants was evaluated by observing sperm motility at exposure times of 5, 10, 15, 20, 25 and 30 min.

2.5. Evaluation of cooling rates

Following the toxicity experiments, 5% and 10% DMSO were chosen for 10 min equilibration, and 5%, 10% and 15% methanol for 20 min equilibration. 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 tong. After sealing, the straws were transferred to a controlled-rate freezer (FREEZE CONTROL® CL-3300; Australia) programmable by computer-based software (CryoGenesis™ V5). Two programmed chamber cooling rates (5 °C/min and 10 °C/min) were tested using a one-step cooling protocol (5 °C to −80 °C). At the end of the program, the straws were retrieved from the cryochamber and plunged into liquid nitrogen for long-term storage. Straws were removed from the liquid nitrogen and thawed at 40 °C for 15 s in water bath and post-thaw motility of the sperm was assessed within 4–6 s after activation with 0.3% NaCl as described above.

2.6. Evaluation of thawing rates

Sperm cryopreserved in 10% DMSO and 10% methanol at 10 °C/min cooling were used for thawing experiments. A water bath was used to heat the samples ($n = 9$) at 20 °C (for 20, 30 and 40 s), 30 °C (10, 20 and 30 s), 40 °C (10, 15 and 20 s) and 50 °C (5, 10 and 15 s). Based on the percent motility, the optimal thawing temperature and duration were evaluated.

2.7. Sperm-to-egg ratio for fertilization

Based on motility, thawing at 40 °C for 15 s was chosen for fertilization trials. To evaluate the sperm-to-egg ratio, eggs were fertilized with sperm (either fresh or thawed), in which the sperm-to-egg ratios were 1.4×10^5:1, 2.3×10^5:1 or 4.1×10^5:1 at room temperature (27 °C). A total of three fertilization trials were conducted, and in each trial, the freshly collected eggs were divided into three aliquot, each containing 5 ml of eggs (1 ml ≈ 1400 eggs). Each group of eggs was fertilized with fresh or thawed (cryopreserved in DMSO or methanol) sperm at designed egg to sperm ratio. The cryopreserved sperm destined for fertilization was stored in liquid nitrogen for 7 –30 days prior to use. The spermatozoa and the eggs were mixed with a hen feather and placed in hatching jars (diameter 0.6 m, height 0.7 m, and capacity of 90 l with 12–14 l/min of water exchange) for incubation of eggs. Fertilization rates were calculated as the number of fertilized eggs in relation to the total number of eggs, and hatching rates were calculated as number of hatchlings obtained from total number of fertilized eggs.

2.8. Statistical analysis

All percent motility values were subjected to arcsine transformation prior to statistical analysis. When time was involved (evaluation of cryoprotectant toxicity), a repeated measurement design was used in the model. When multiple variables were involved (evaluation of cooling rates; evaluation of thawing rates, fertilization and hatching), treatment effects were analyzed using one-factor, or two-factor Analysis of Variance (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).

3. Results

3.1. Evaluation of cryoprotection toxicity

When activated with 0.3% NaCl, 90% of the fresh sperm showed forward movement before equilibration with the cryoprotectants. After equilibration, motility was significantly affected by the time ($F_{5, 240} = 156.01$; $P = 0.001$) and the interaction between the effects (time × cryoprotectant × concentration; $F_{10, 240} = 24.85$; $P = 0.001$). When equilibrated in 5% DMSO, the diluted samples maintained higher percent motility at all time periods tested than when in 10% or 15% DMSO (Table 1).

3.2. Evaluation of cooling rates

Based on the previous experiment, 15% DMSO was eliminated from further consideration because of its acute toxicity. There were significant differences in post-thaw motility under different cooling rates ($F_{1, 110} = 13.34$; $P = 0.001$), cryoprotectant types ($F_{1, 110} = 29.14$; $P = 0.001$), cryoprotectant concentrations ($F_{1, 110} = 319.66$; $P = 0.001$) and their interactions ($F_{2, 110} = 5.06$; $P = 0.026$). Of the cooling rates tested, 10 °C/min yielded significantly ($P = 0.011$) better post-thaw motility when equilibrated with 10% DMSO (66 ± 5%) and 10% methanol (60 ± 9%) (Table 2).

3.3. Evaluation of thawing rates

There were significant differences in post-thaw motility among the thawing temperatures and thawing durations ($F_{11, 71} = 58.95$; $P = 0.001$) (Table 3). The highest motility was observed for both cryoprotectants at 40 °C for 15 s.

3.4. Fertilization and hatching

No significant differences were observed among the fertilization and hatching rates for the cryoprotectants tested. Significant differences were observed ($F_{2, 72} = 209.84$ for fertilization and $F_{2, 72} = 352.06$ for hatching rates; $P = 0.001$)

Table 1
Mean (±SD) of equilibration motility of *L. calbasu* sperm with two cryoprotectants (dimethyl sulfoxide and methanol) at final concentrations of 5%, 10% and 15% in modified Alsever's solution at an osmolality of 296 mOsmol kg^{-1} ($n = 3$). The samples were placed in refrigerator (4 °C) during study.

Cryoprotectant	Concentration	Equilibration time (min)					
		5	10	15	20	25	30
DMSO	5%	90 ± 0^{a1}	90 ± 0^{a1}	90 ± 3^{a1}	89 ± 0^{a1}	88 ± 4^{ab1}	84 ± 7^{b1}
	10%	84 ± 5^{a2}	78 ± 7^{b34}	73 ± 5^{b2}	62 ± 6^{c2}	57 ± 10^{c4}	42 ± 9^{d3}
	15%	46 ± 5^{a3}	28 ± 7^{b5}	19 ± 8^{c3}	2 ± 4^{d3}	–	–
Methanol	5%	90 ± 0^{a1}	84 ± 5^{ab2}	86 ± 5^{ab1}	83 ± 10^{b1}	82 ± 5^{b2}	82 ± 7^{b1}
	10%	83 ± 6^{a2}	74 ± 5^{b4}	73 ± 5^{bc2}	72 ± 4^{bc2}	70 ± 0^{bc3}	69 ± 2^{c2}
	15%	85 ± 4^{a2}	81 ± 8^{a23}	70 ± 7^{b2}	72 ± 3^{b2}	70 ± 0^{b3}	69 ± 2^{b2}

Values with different letters in superscript are significantly different within a row and values with different numbers in superscript are significantly different within a column.

Table 2
Mean (±SD) and minimum-maximum motility of *L. calbasu* sperm ($n = 4$) before and after cryopreservation using different concentrations of DMSO and methanol as cryoprotectants with two cooling rates (5 and 10 °C/min) from 5 to −80 °C (equilibration time 10 min for 5% and 10% DMSO and 20 min for 5%, 10% and 15% methanol). The sperm were stored in liquid nitrogen for 7–30 days before thawing.

Cryoprotectant	Concentration	Cooling rate (°C/min)	Equilibration motility (%)	Post-thaw motility (%)
DMSO	5	5	83 ± 5^{a} (80–90)	23 ± 5^{e} (15–30)
		10	80 ± 6^{abc} (90–70)	14 ± 5^{f} (10–20)
	10	5	71 ± 9^{d} (60–80)	41 ± 8^{c} (30–50)
		10	72 ± 4^{d} (70–80)	66 ± 5^{a} (40–70)
Methanol	5	5	85 ± 7^{a} (70–90)	20 ± 0^{e} (0–20)
		10	80 ± 6^{abc} (70–90)	3 ± 5^{g} (0–10)
	10	5	82 ± 7^{ab} (70–90)	30 ± 0^{d} (30–30)
		10	75 ± 5^{cd} (70–80)	60 ± 9^{b} (50–70)
	15	5	78 ± 6^{bc} (70–90)	40 ± 9^{c} (30–50)
		10	77 ± 5^{bcd} (70–80)	37 ± 10^{c} (30–50)

Values with different superscripts within a column are significantly different.

Table 3
Mean (±SD) and minimum-maximum post-thaw motility of *L. calbasu* sperm thawed at four temperatures for different durations (s) ($n = 5$). The samples were stored in liquid nitrogen for 6–9 months before thawing. The cryopreserved spermatozoa were activated with 0.3% NaCl (96 mOsmol kg^{-1}).

Temperature (°C)	Duration (s)	Post-thaw motility (%)	
		10% DMSO mean ± SD (min.–max.)	10% methanol mean ± SD (min.–max.)
20	20	7 ± 5 (0–10)^{d}	10 ± 9 (0–20)^{e}
	30	46 ± 7 (35–50)^{b}	39 ± 12 (20–50)^{c}
	40	66 ± 5 (60–70)^{ab}	63 ± 11 (50–75)^{ab}
30	10	11 ± 7 (0–20)^{d}	10 ± 9 (0–20)^{e}
	20	53 ± 5 (50–60)^{b}	50 ± 0 (50–50)^{bc}
	30	64 ± 5 (60–70)^{ab}	65 ± 5 (60–70)^{a}
40	10	53 ± 8 (45–65)^{b}	50 ± 4 (45–55)^{bc}
	15	75 ± 5 (70–80)^{a}	74 ± 7 (70–80)^{a}
	20	67 ± 5 (60–70)^{ab}	62 ± 7 (55–70)^{ab}
50	5	18 ± 8 (10–30)^{c}	18 ± 12 (10–35)^{d}
	10	68 ± 4 (60–70)^{ab}	63 ± 5 (60–70)^{ab}
	15	43 ± 5 (40–50)^{b}	43 ± 5 (40–50)^{c}

Values with different superscripts within a column are significantly different.

Table 4
The effect of sperm-to-egg ratio on fertilization in *L. calbasu*. Eggs were fertilized at room temperature (27 °C) using fresh and cryopreserved spermatozoa. The samples were stored in liquid nitrogen for 7–30 days prior to thawing. The cryopreserved and fresh spermatozoa were activated with 0.3% NaCl (96 mOsmol kg^{-1}) and used with eggs from three females.

Attributes	Sperm-to-egg ratio	Fertilization (%) Mean ± SD (min.–max.)	Hatching (%) Mean ± SD (min.–max.)
10%	1.4×10^5:1	47 ± 4^{d} (43–52)	33 ± 3^{c} (28–36)
DMSO	2.3×10^5:1	55 ± 3^{c} (50–60)	42 ± 3^{b} (38–46)
	4.1×10^5:1	65 ± 3^{b} (60–71)	45 ± 2^{b} (42–49)
10%	1.4×10^5:1	45 ± 4^{d} (40–53)	30 ± 2^{c} (27–37)
methanol	2.3×10^5:1	55 ± 3^{c} (50–59)	43 ± 4^{b} (37–49)
	4.1×10^5:1	63 ± 4^{b} (59–69)	45 ± 3^{b} (40–49)
Fresh	1.4×10^5:1	62 ± 4^{b} (57–70)	45 ± 3^{b} (41–49)
	2.3×10^5:1	75 ± 4^{a} (70–81)	64 ± 4^{a} (60–70)
	4.1×10^5:1	79 ± 3^{a} (74–83)	66 ± 3^{a} (61–71)

Values with different superscripts within a column are significantly different.

between cryopreserved and fresh sperm as well as between different sperm-to-egg ratios ($F_{2,72} = 1.66$ for fertilization and $F_{2,72} = 230.96$ for hatching rates; $P = 0.001$). Highest fertilization of eggs were achieved at 4.1×10^5:1, and highest hatching at 2.3×10^5 as well as 4.1×10^5:1 sperm-to-egg ratio for cryopreserved *L. calbasu* sperm (Table 4).

4. Discussion

To our knowledge, this is the first comprehensive report on sperm cryopreservation of *L. calbasu*. The sperm cryopreservation protocol developed in the present study is based on collection and dilution in readily available diluents, optimal equilibration in cryoprotectants, optimization of cooling rate, determination of thawing temperature and duration, and fertilization of eggs.

Motility of sperm outside the testis is typically inhibited by use of an immobilizing solution that has similar osmolality to the seminal plasma (Billard et al., 1995). Sperm collected in modified Alsever's solution (osmolality of 296 mOsmol kg^{-1}) conforms with the seminal plasma osmolalities typically observed for freshwater fishes (230–346 mOsmol kg^{-1}) (Alavi and Cosson, 2006), and thus immobilized *L. calbasu* sperm. Outside of the testis, freshwater fish sperm respond to a hypo-osmotic shock for induction of motility (Morisawa and Morisawa, 1986) or motility can be initiated by reducing the concentration of potassium ions as in salmonids (Morisawa et al., 1983; Cosson, 2004). Therefore, 0.3% NaCl (96 mOsmol kg^{-1}), a hypo-osmotic solution was used as activation media to trigger sperm motility.

In the toxicity experiment, percent motility was higher at longer time intervals of *L. calbasu* sperm when equilibrated with 5% DMSO than with 10% and 15% DMSO. This is because of the toxicity or osmotic effects of DMSO at higher concentrations, and as such there should be an optimal balance between toxicity and the ability to protect sperm cells during cryopreservation. Sperm of the red snapper (*Lutjanus argentimaculatus*) maintained higher percent motility at longer time intervals when equilibrated with 5% DMSO than with 10%, 15% and 20% DMSO (Vuthiphandchai et al., 2009). In this study, when equilibrated with methanol, motility of the sperm was not reduced at different concentrations (5%, 10% and 15%) after 30 min of equilibration. As such methanol was less toxic to sperm, and enabled longer equilibration. Methanol was also found to have reduced toxicity compared to DMSO for *Puntius sarana* sperm at each of the concentrations and equilibration times tested (Nahiduzzaman et al., 2011).

In the present study, two different single-step cooling rates (5 and 10 °C/min) and different cryoprotectant concentrations were tested. During cooling, ice crystal nucleation within cells generally occurs at above −40 °C (Friedler et al., 1988) and when this temperature has been passed without damage, cells can be safely stored in liquid nitrogen. The success of a cooling rate depends on several factors such as the cell type and size, membrane composition, cryoprotectant type and concentration, equilibration time, and on the interactions among these factors (Yao et al., 2000). Therefore, we sought to balance these factors when *L. calbasu* sperm were cooled at 10 °C/min after 10 min equilibration with 10% DMSO or methanol. The nominal rate of 10 °C/min can be considered a medium cooling rate and has been successful for other fish species, including razorback sucker *Xyrauchen texanus* (Tiersch et al., 1998), African catfish *Clarias gariepinus* (Urbanyi et al., 1999) sea bream *Sparus aurata* (Fabbrocini et al., 2000), sea bass *Dicentrarchus labrax* (Sansone et al., 2001) and green swordtail *Xiphophorus helleri* (Huang et al., 2004).

In the thawing experiment, highest percent motility was found after heating at 40 °C for 15 s, because it likely prevented recrystallization most effectively. From this, a change of 5 s in duration or a change of 10 °C in the temperature led to reductions in post-thaw motility. Successful thawing of cryopreserved sperm has been found in cyprinid fishes within the temperature range of 30–40 °C (Magyary et al., 1996; Ramirez-Merlano et al., 2011). In general, protocols can be optimized in a range of thawing temperature from 0 to 60 °C for a variety of species considering sample volume, container type (e.g., volume, materials, and geometry) as these can affect the rate of heat transfer (Tiersch, 2011).

Cryopreserved sperm produced viable offspring of *L. calbasu* although at a 10–20% lower rate of fertilization and hatching in the same trial compared to the rates obtained using fresh sperm. In previous studies, higher numbers of thawed sperm were required to fertilize the eggs of common carp, *Cyprinus carpio* (Lubzens et al., 1997), *Pangasius hypophtalmus* (Kwantong and Bart, 2003) and *Misgurnus anguillicaudatus* (Yasui et al., 2009), whereas equivalent hatching rates were obtained for silver carp, *Hypophthalmicthys molitrix* (Alvarez et al., 2003) when appropriate comparisons are made between fresh and cryopreserved sperm. Overall, the optimal number of sperm required to fertilize eggs varies and can be species specific. In this study, when cryopreserved sperm were used, highest fertilization of eggs were observed at 4.13×10^5:1 and highest hatching at 2.3×10^5 as well as 4.13×10^5:1 sperm-to-egg ratio for *L. calbasu*. The sperm cryopreservation studies reported on sperm-to-egg ratios for fertilization of the eggs, 1.0×10^5 sperm per egg was reported for *Siniperca chuatsi* (Ding et al., 2009), 2.5×10^5 sperm per egg for *Ictalurus punctatus* (Tiersch et al., 1994), and 4.2×10^6 sperm per egg for *Pangasius gigas* (Mongkonpunya et al., 1995).

In summary, *L. calbasu* sperm were cryopreserved after collection in Alsever's solution, equilibration with 10% DMSO (10 min) or 10% methanol (20 min), with a cooling rate of 10 °C/min from 5 to −80 °C, and storage in liquid nitrogen. The preserved sperm were retrieved from liquid nitrogen and thawed at 40 °C for 15 s to fertilize eggs after gamete activation with 0.3% NaCl. This protocol would contribute to the commercialization of cryopreserved fish sperm for hatchery operation and conservation of genetic material of *L. calbasu*. The growth rate of larvae produced from cryopreserved sperm was the same as for fresh sperm for *I. punctatus* (Tiersch et al., 1994). There are few studies worldwide that compared the growth rate (Chereguini et al., 2002) and percentage of malformed larvae (Rideout et al., 2003; Miskolczi et al., 2005) obtained from cryopreserved and fresh sperm. Therefore, prior to starting the commercial application or conservation action

of crypreserved sperm of any fish species at a large scale, larval quality issues should be studied.

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