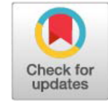
Pakistan J. Zool., pp 1-11, 2022. DOI: https://dx.doi.org/10.17582/journal.pjz/20220330110350

Effect of Inducement, Dilution Ratio and Freezing Rate on the Quality of *Cyprinus carpio* (Linnaeus, 1758) Spermatozoa



Kenyum Lollen[1], Judith Betsy C[1]*, Cheryl Antony[2] and Stephen Sampath Kumar J[3]

[1]*Department of Aquaculture, Fisheries College and Research Institute, Tuticorin, Tamil Nadu, India.*
[2]*Department of Aquaculture, Dr. M.G.R. Fisheries College and Research Institute, Ponneri, Tamil Nadu, India.*
[3]*Directorate of Sustainable Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam, Tamil Nadu, India.*

Article Information
Received 30 March 2022
Revised 15 September 2022
Accepted 21 October 2022
Available online 12 January 2023 (early access)

Authors' Contribution
KL execution of the experiment, data analysis, manuscript writing and reviewing. CJB conceptualization, design and execution of the experiments, data analysis, manuscript writing and reviewing. CA manuscript correction. JSSK conceptualization and design of the experiment. All authors contributed critically to the drafts and gave final approval for publication.

Key words
***C. carpio*, Inducement, Dilution ratio, Freezing rate, Fertilization**

ABSTRACT

Cyprinus carpio is a widely cultured species and exploitation of natural stock due to increased demand in production can be averted by hormonal inducements which further affects the quality of gametes. Cryopreservation can aid in the conservation of gene pool and bestow superior quality sperm. Freezing and thawing rate are the most critical factor in the cryopreservation of milt. In the present study, variability between spermatological parameters of induced and non-induced *C. carpio* was documented for a period of 7 months. Further, the non-induced milt was stored for 24 h at three dilution ratio (1:10, 1:20 and 1:40) and spermatological parameters were analysed. Then the milt was cryopreserved with the best dilution ratio using 3 freezing protocols with two step cooling profile in programmable freezer. The spermatological properties of milt obtained from non-induced fishes were superior which was further used for short term preservation. When experimented with dilution ratio, the highest motility duration of 74.3±2.16 s was obtained at 1:10 dilution ratio. The best results on motility duration (62.28 ± 2.12 s), fertilizing ability (77.3±1.63 %) and hatching rate (61.6±2.44 %) was recorded when freezing protocol I was followed and the values were statistically significant. Hence the results revealed that for successful cryopreservation of *C. carpio* milt, use of non-induced milt at 1:10 dilution ratio using slow freezing rate will give the best results in terms of motility, fertilization and hatching rate.

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INTODUCTION

The total world fisheries and aquaculture production has reached 179 million tonnes in 2018 which was recorded as the highest of all time and its value has been estimated at USD 401 billion (FAO, 2020). The aquaculture sector was the main driver that has helped the increase in production of aquatic animals. The global aquaculture production was dominated by carps. Common carp (*Cyprinus carpio*) is a widely cultured freshwater species and is an exhaustively researched Cyprinid. It is one of the most important fish species in global aquaculture with a share of 7.7% (4189.5 thousand tonnes) to the total global freshwater aquaculture production (FAO, 2020).

However, due to the emergence of intensive aquaculture of carps and development of commercial aquaculture, there is a need to acquire high quality seed for grow-out of marketable size under a given period of time. As it is difficult to meet such a high demand of seed through the natural breeding of carp, hormonal manipulations are used as a management tool to enhance the efficiency of egg production, increase spermiation and facilitate hatchery operations (Mylonas *et al*., 2010). The seed production of cultivable carps are mainly based on the induced breeding through hormones like carp pituitary extract, luteinizing hormone releasing hormone, human chorionic gonadotropin, Wova-FH, Ovaprim and Ovatide and the success largely depends upon the availability of well-maintained broodstock (Gupta *et al*., 1990). Though hormonal inducement can make the fish to breed, the sperm quality plays an important role in determining the fertilization success and it is known that the hormonal

* Corresponding author: betsy@tnfu.ac.in
0030-9923/2022/0001-0001 $ 9.00/0

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inducement affect the milt quality and motility of spermatozoa (Clemens and Grant, 1965). Belova (1981) stated that milt obtained from captive fish is low in volume and high in sperm concentration but the milt obtained as a result of hormonal stimulation had increased milt volume and low sperm concentration.

Although hatchery produced seeds are available for *C. carpio*, the gamete quality, purity and genetic quality of the seeds is a big question because of continuous hormonal manipulations, inbreeding and unintentional intraspecific hybridization practiced by the hatchery operators. It is, therefore, necessary to conserve the gene pool of wild stocks for quality brood production (Khan *et al*., 2015). In the above light, cryopreservation of fish gametes and application in the seed production is considered to be the simplest and most inexpensive method to preserve genomes that can be used to conserve the wild gene pool (Rafiquzzaman *et al*., 2007). First experiments on the cryopreservation of common carp milt were reported by Moczarski (1977) and the topic has been studied extensively since then. Despite successful cryopreservation of more than 200 fish species (Tsai and Lin, 2012), the quality of post-thaw spermatozoa are reported to deteriorate remarkably which straight away affects the rate of fertilisation and hatching (Liu *et al*., 2007).

The freeze-thaw step is the most crucial aspect of cryopreservation which needs to be optimized in order to decrease the impairment of spermatozoa occurred during the freezing process (Yoon *et al*., 2015). Several authors (Cloud and Patton, 2008; Dziewulska and Domagala, 2013) observed increased efficiency of spermatozoa when an effective dilution ratio in combination with cryoprotectants was manoeuvred during cryopreservation. Lahnsteiner *et al*. (2003) mentioned that at a ratio of 1:10, the osmolality of the extender–water mixture was high enough to stabilize sperm viability. He also stated that too low dilution ratio did not activate full sperm motility and too high ratios resulted in insufficient low sperm concentrations in the fertilization solution. Contrary to that, Linhart *et al*. (2000) reported 1:25 ratio as the optimal ratio of milt to fertilization medium and Magyary *et al*. (1996) found 1:20 as the optimal ratio for *C. carpio*. Sultana *et al*. (2009) observed that the best ratio for the milt and cryodiluents for the *C. carpio* spermatozoa was at 1:9 considering both pre-freeze and post-thaw motilities of spermatozoa.

The conventional freezing methods are known to have ill effects called cryoinjuries on the spermatozoa cryopreserved (Bozkurt *et al*., 2005). Thus, the choice and concentration of cryoprotectants, rate of freezing and cooling must be optimized for each species as the basis for any protocol development and successful cryopreservation process (Agrawal, 2011). The formation of intracellular ice crystal or spermatozoa dehydration can be circumvented by following an appropriate cooling rate during freezing (Cloud and Patton, 2008). Hence in the present study, the variations in the spermatological properties of induced and non-induced fishes were documented. Also, the best dilution ratio along with freezing rate was studied to minimise cryo injuries during the cryopreservation of *C. carpio* milt.

MATERIALS AND METHODS

Experimental fish

Adult male *C. carpio* brooders (50 nos) with an average body weight (ABW) of 468±0.5 g were procured and maintained in 2 cement tanks of 9000 l capacity each. During the period of rearing, the fishes were provided with supplementary feed @ 2-3% BW. The faecal matters and uneaten feeds were siphoned out daily from the tanks. The water was exchanged once in 2 days at 70% level.

Milt collection

Milt from male fishes was collected from the donor with mild oozing milt. Milt collection was done by gentle stripping as described by Lubzens *et al*. (1997). The genital pore region was cleaned with absorbent cotton and double ply tissue paper to remove any moisture or mucus in that area. The evacuation of urine was done by mild gentle press near the genital pore anteriorly, that could also press out the faecal matter if any to avoid contamination of the milt and the milt was collected in a sterile, pre-labelled vial (Betsy *et al*., 2019).

Experimental design

The study consisted of three independent trials. The aim of Trial 1 was to evaluate the effect of inducement on the spermatological properties. Trial 2 was performed to assess the effect of different dilution ratios on the milt quality before cryopreservation. In Trial 3, the influence of different freezing rate was assessed with best dilution ratio based on (i) sperm motility and (ii) fertilizing capacity.

Effect of hormonal inducement on spermatological properties

After acclimatization of fishes, once in a week, 5 male brooders were administered with single dose of Wova-FH (Biostadt Agrisciences, Wockhardt Life Sciences, Mumbai, India) @ 0.5 ml/kg BW using a 1 ml graduated syringe intramuscularly at an angle of 45° in the dorsal side (Basudha *et al*., 2017). The brooders induced with hormone was maintained in FRP tanks till milt collection.

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Milt samples were collected from both induced and non-induced fishes. During every collection, 5 number of brooders were used each for induced and non-induced treatment. Milt was collected from induced fishes after 12 h of hormone administration.

Effect of dilution ratios on the milt quality

Based on the results obtained in the first trial, milt collected from non-induced fishes alone was used to evaluate the effect of dilution ratio. Milt from 10 fishes were collected for each treatment and pooled together. The collected milt was diluted with Freshwater Fish Saline (FWFS) (7.5g NaCl, 0.2g KCl, 0.2g $NaHCo_3$, 0.2g $CaCl_2$ per 100 ml of DW) as extender and DMSO at 10% (v/v) as cryoprotectant. Hence the ratio of extender to cryoprotectant was 9:1 (Betsy *et al*., 2017). All chemicals were of AR grade purchased from Merck, Germany. The milt dilution was performed inside the cold handling unit in 10 ml glass beakers. The freshly collected milt was diluted with extender and cryoprotectant at three different dilution ratios viz., 1:10, 1:20 and 1:40. Extender was added to the sample drop by drop during constant gentle mixing to avoid an osmotic shock of spermatozoa by cryoprotectant (Boryshpolets *et al*., 2017). The diluted spermatozoa was refrigerated at 4 °C for 24 h after which the spermatological properties were evaluated.

Effect of freezing rate on the milt quality

For the purpose of 3rd trial, milt was again collected from 10 fishes for each treatment and pooled together and was diluted with the best dilution ratio obtained from Trial 2. The diluted milt was equilibrated for a period of 10 min at 4°C in the cold handling unit. After equilibration, the diluted milt was loaded in cryovials of 2 ml volume. The loaded cryovials were transferred to controlled rate freezer (PLANER, Kryo 560-16) programmable by Planer's MRV controller system. Three sets of three step freezing protocol (FP) were used such as, FP I: 5°C to -4°C (at the rate of 4°C/min) (Ramp 1 min) and from -4°C to -80°C (at the rate of 10°C/min) and held for 10 min and direct transfer to liquid nitrogen (LN_2); FP II: 5°C to -4°C (at the rate of 5°C/min) (Ramp 1 min) and from -4°C to -80°C (at the rate of 10°C/min) and held for 10 min and direct transfer to liquid nitrogen LN_2 and FP III: 5°C to -4°C (at the rate of 10°C/min) (Ramp 1 min) and from -4°C to -80°C (at the rate of 10°C/min) and held for 10 min and direct transfer to liquid nitrogen LN_2.

Once the curve programming was complete and the temperature reached -80°C, the straws were immediately transferred to a BA11 Cryocan (IBP, India) for storage at -196° C in canister containing goblet. The BA11 Cryocan was maintained with LN_2 to a depth of 20 to 23 cm from the bottom that was checked every week with the help of a dip stick. The quality of cryopreserved spermatozoa maintained in the BA11 cryocan with LN_2 was analyzed once in 10 days for a period of 30 days (Judycka *et al*., 2017).

During each sampling, cryopreserved vials were taken out from the BA11 cryocan and thawed at 25°C for 1-2 min as recommended by Lubzens *et al*. (1997) in serological water bath. The vials were thawed rapidly to avoid recrystallization (Lahnsteiner *et al*., 2000). After thawing, the water was wiped off the vials and the sample was taken for observation. The sample was examined for the parameters as that of the pre- frozen milt and the data obtained was recorded.

Assessment of sperm motility and fertilizing ability

Sperm motility

The collected milt was analysed under phase contrast microscope (NIKON E360) at X 200 magnification for its motility duration which was evaluated through the observation of their motility by placing 1 µl of milt sample and 10 µl of tap water on glass slide and observing under microscope (Akcay *et al*., 2004). The motility assessment was carried out before and after dilution. The motility score was assessed following Betsy and Kumar (2014).

Sperm density

The density of spermatozoa was determined by counting in a standard hemocytometer with an area of 1/25+1/400 mm² and depth of 0.1 mm (Naubaeur, Germany). The sperm was diluted 100 folds with freshwater fish saline as described by Betsy *et al*. (2019). A droplet of the diluted milt was placed on a haemocytometer slide and counted using light microscope.

The number of spermatozoa per ml of milt was determined for each sample by using the following formula given by Aramli *et al*. (2013) and spermatozoa density was expressed as × 10^9 cells/ml.

$$\text{Sperm density (per ml)} = \frac{\text{Number of counted sperm}}{\text{Area (mm2) x Chamber depth (mm) x Dilution ratio}} \times 1000$$

Percentage of live and dead spermatozoa

The percentage of live and dead spermatozoa was assessed using Eosin-Nigrosin stain as described by Chutia *et al*. (1998). On grease free glass slides, 1 µl of diluted milt was mixed with 1 µl of Eosin-Nigrosin stain placed on the corner of the slide. With the help of cover glass, the milt and the stain were dragged front and back for proper mixing. The smears were dried at 40°C in hot plate (5 MLH-DX, Remi Equipment) for 10 to 20 s. The percentage of live and dead spermatozoa was calculated by the concept of stain exclusion by the living cells. This was

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ascertained by microscopic observations (Nikon E360).

Fertilization ability

Dry method of *in vitro* fertilization was practiced following Sultana *et al*. (2009) and Aliniya *et al*. (2013). Fresh milt was collected from the fishes and diluted with same diluents and dilution ratios used for frozen sperm to minimize error. Eggs that were fertilized with the fresh milt were maintained as Control. Fertilisation was done in dry plastic dishes (Betsy *et al*., 2019). Cryopreserved spermatozoa were thawed for 2 min and added over the eggs immediately and gently mixed with feather. After thorough mixing, water was added to the milt egg mixture and mixed for one minute (Sultana *et al*., 2009). Then the eggs were transferred to nylon filaments. The eggs that were attached with the filaments were placed in hapa for incubation. Fertilization parameters such as fertilization rate (Brommage and Cumaranatunga, 1988) and hatching rate (Hanjavanit *et al.,* 2008) were studied for all the samples. The calculation for fertilization and hatching rate was done as follows:

Fertilization rate = [Number of fertilized eggs/ Total eggs] × 100

Hatching rate = [Number of healthy fertilized egg/ Number of fertilized eggs] × 100

Statistical analysis

Data were obtained in triplicates and expressed as mean ± SD. Analysis of statistical significance between treatments was performed using one way ANOVA ($p<0.05$) and paired sample t test ($p<0.001$). All analysis were carried out using SPSS for windows version 22.0.

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RESULTS AND DISCUSSION

Effect of hormonal inducement on spermatological parameters of non-induced milt

Motility duration

The motility duration of spermatozoa obtained from non-induced milt exhibited high variations. The highest mean motility duration (79±8.28 s) was obtained during February, 2021 (Table I). Uniform mean motility duration of 78 ±2.49 s and 78 ±6.23 s was documented during the month of November and December, 2020, respectively. The milt collected during the months of January, March and May 2021 reflected mean motility duration of 77±7.84 s, 75±3.74 s and 68±1.29 s, respectively (Table I). There was a decreasing trend of motility duration from February to April. The lowest mean motility duration was recorded during the month of April 2021 (62±3.29 s) (Table I). These values complies with that of Rahman *et al*. (2011) for fresh milt of *Hypophthalmichthys molitrix* and Bighead carp (*H. nobilis*) which had mean motility duration of 95.78±0.49 s and 95.11±0.35 s, respectively during the early spawning seasons. According to Yaron *et al*. (2009), the early spawning season of *C. carpio* was in the month of February which corresponds with the present work where the highest motility duration was obtained during the month of February. The values obtained was in accordance with Bozkurt *et al*. (2008) who reported 67.68±4.32 s as average motility duration in grass carp (*Ctenopharyngodon idella*). The motility score recorded in all samples of non-induced milt was 10.

The fluctuating motility durations and concentrations at the beginning and end of spawning may be due to factors such as age, length and weight and rearing conditions of brood stock (Faruk and Zafer, 2005). It is also to be noted that the repeated use of the same brooder over seasons, nutritional requirement, stocking density, and environmental changes together or individually may also affect the sperm quality that changes between spawning seasons (Rahman *et al*., 2011). The parameters of milt varies between conspecific males, between species and across the reproductive season (Rahman *et al*., 2011).

Sperm density

The highest spermatozoa density of 3.25 x10^9 Cells/ml was obtained during the month of January 2021 whereas, the lowest value of 1.96x10^9 Cells/ml was recorded during May 2021 (Table I). This is in accordance with Rahman *et al*. (2011) who reported higher sperm density of 3.15 x10^9 Cells/ml during early spawning seasons for Silver carp and lower density of spermatozoa of 2.42 x10^9 Cells/ml during late spawning season. Although May month was not the late spawning season for common carp, in the present study lowest mean motility duration was recorded during May which could be due to other factors such as age of the brooder and rearing condition (Faruk and Zafer, 2005). Milt collected during February and April 2021 exhibited almost similar spermatozoa density (Table I).

The observed difference in the sperm concentration across spawning seasons could have resulted from discontinuous spermatogenesis (Piros *et al*., 2002), may be due to changing endocrine conditions that affect spermatozoa maturation (Alavi *et al*., 2008). The spermatozoa concentration were found to decrease for rainbow trout, *Onchorhynchus mykiss* (Buyukhatipoglu and Holtz, 1984) and *C. carpio* (Christ *et al*., 1996) as the spawning season advanced.

Effect of hormonal inducements on spermatological parameters of induced milt

Motility duration

The motility duration of spermatozoa significantly

changed over the study period and there was no particular increasing or decreasing trend in motility duration. From Table I, it can be observed that the highest mean motility duration obtained for induced *C. carpio* spermatozoa was 70±5.43 s during the month of April 2021. The milt collected during the month of November 2020 and February 2021 exhibited similar mean motility duration of 63±3.09 s and 63±2.62 s, respectively. The lowest mean motility duration of 59±7.03 s was recorded during January 2021 (Table I). The motility score recorded in all samples of induced milt was 8.

Table I. Spermatological parameters of induced and non-induced milt.

Treatment	Mean motility duration (s)	Sperm density ($x10^9$ Cells/ml)
Induced milt		
November,2020	63 ±3.09^e	1±0.06^g
December, 2020	69±5.09^b	2.14±0.36^c
January, 2021	59±7.03^g	2.5 ±0.69a
February, 2021	63±2.62^e	1.75± 0.49^f
March,2021	67±2.86^d	2.21± 0.60^b
April, 2021	70±5.43^a	1.89± 0.04^d
May, 2021	68±0.81^c	1.8 ±0.16^e
Non-induced milt		
November,2020	78 ±2.49^b	2.96± 0.10^c
December, 2020	78 ±6.23^b	2.18±0.04^f
January, 2021	77±7.84^d	3.25±0.08^a
February, 2021	79±8.28^a	2.21±0.03^e
March,2021	75±3.74^e	3.05±0.13^b
April, 2021	62±3.29^g	2.25±0.02^d
May, 2021	68±1.29^f	1.96±0.05^g

Data expressed as Mean ± SE (n=5, r=3); Mean values in same column with different subscript differ significantly ($p<0.05$) between months. One way ANOVA was used following Duncan multiple range test in SPSS-22.0

The findings on motility duration in the present study corroborates with the results reported by Verma *et al.* (2009) for silver carp (*Hypopthalmichthys molitrix*), who had reported 75±3.5 s as the highest mean motility duration during the month of June when induced with Ovaprim. The variation in motility duration throughout the study period can be attributed to the changing season, temperature, pH, osmolality (Lahnsteiner *et al.*, 1998; Cosson *et al.*, 2008) and are found to fluctuate between different males depending on the ripeness during the spermiation period (Billard, 1986). The motility duration in several Cyprinids were reported to be in the range of 90-120 s (Suzuki, 1959).

Sperm density

The sperm density fluctuated throughout the course of study period. The highest spermatozoa density ($2.5x10^9$ Cells /ml) was obtained during the month of January 2021 (Table I). Almost similar spermatozoa density of 1.89 $x10^9$ Cells /ml and 1.8 $x10^9$ Cells /ml was obtained in the milt samples collected during April 2021 and May 2021 respectively. However, during November 2020 the density of spermatozoa was recorded to be least with $1x10^9$ Cells / ml (Table I).

This is in accordance with the work of Nahiduzzaman *et al*. (2014) who reported the highest mean sperm density of 2.28 $x10^9$ Cells /ml in *C. carpio* during the month of December and the lowest density of 1.08 $x10^9$ Cells /ml during March when induced with carp pituitary. Alavi *et al*. (2008) has demonstrated a similar seasonal change of sperm concentration (18.81 $\times10^9$ Cells/ml in March to 12.45×10^9 Cells/ml in May) in barbel (*Barbus barbus)*. The difference in the concentration of sperm density across the seasons might be related to gonadal development and maturation, which is regulated by change in climate, day length and food supply, hormonal stimulation methods, stress, environmental conditions and age of the brood fish (Piros *et al*., 2002).

Effect of dilution ratios on the milt quality

Motility duration

The initial motility duration of spermatozoa after dilution was 79.6±1.63 s, 77.3±3.74 s and 75.6±2.16 s at 1:10, 1:20 and 1:40 dilution, respectively. When the milt was evaluated after 24 h of short-term preservation, the highest motility duration of 74.3±2.16 s was obtained at 1:10 dilution ratio. The second highest motility duration (72.0±1.63 s) was observed in 1:20 dilution, whereas the lowest motility duration of 70.3±2.82 s was obtained at 1:40 dilution. The values obtained were statistically significant ($p<0.001$) when analysed using paired sample t test.

This result is in accordance with the reports of Basavaraja and Hegde (2004) who mentioned highest post-thaw motility duration of 77 s during the cryopreservation of deccan mahseer (*Tor Khudree)* milt at 1:10 dilution ratios. Muchlisin *et al*. (2004) reported decreased motility duration with increase in dilution ratio (1:20, 1:30 and 1:40) and highest post-thaw motility duration of 71 s was observed at lower dilution of 1:20 in tropical bagrid fish (*Mystus nemurus*) when the milt was stored for 24 h in freezer. Alawi *et al.* (1995) reported a decreasing trend in motility duration as the dilution ratio increased. This may be because, at higher dilution ratios, the swimming

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velocity of spermatozoa and the percentage of linearly motile spermatozoa were decreased (Suquet *et al.*, 2000; Lahnsteiner, 2007) due to removal of protective components of seminal plasma. Chauvaud *et al.* (1995) reported the protective action of proteins in the diluent on sperm motility and prevention of sperm aggregation in turbot spermatozoa. The motility and fertility of deep frozen spermatozoa of *C. carpio* were reported to be significantly improved when the dilution ratio was reduced from 1:100 to 1:2 (Cognie *et al*., 1989).

The other explanations regarding decreased motility duration of spermatozoa with increased dilution involves ionic composition of the diluent used as it affects the sperm motility (Erdahl and Graham, 1987; Erdahl *et al*., 1987). K^+ ions are major constituents of carp seminal plasma and were found to increase the viability and speed of spermatozoa (Morisawa *et al*., 1983). Various authors reported increase in sperm motility in carp, goldfish and crucian carp when the level of K+ ions were higher in seminal plasma and concluded that it may help maintain the spermatozoa viability as the milt is diluted (Clemens and Grant, 1965; Grant *et al*., 1969).

Furthermore, Bozkurt *et al.* (2005), reported higher motility duration of 360.16±177 s when the milt of *C. carpio* was stored for short period of time at 1:3 dilution ratio. According to Saad *et al.* (1988), when the *C. carpio* milt was stored for short period of time at 4 °C, they obtained higher percentage of motile spermatozoa up to 2 days which rapidly decreased after 6-8 days. Contrary to this, Ani and Jayaprakas (2013) obtained very low motility duration of 18.6 ± 0.51 s and 45 ± 0.31 s for slender rasbora *(Rasbora daniconius*) and filament barb (*Puntius filamentosus*) respectively, when the milt was stored at 4°C for 24 h.

Percentage of live and dead cells

When the *C. carpio* milt was analysed after short term preservation, the highest percentage of live cells observed was 85% at 1:10 dilution ratio, while at dilution ratio of 1:20 and 1:40, the live cells recorded was 79% and 76%, respectively which falls in line with the reports of Betsy and Kumar (2016), who reported 80% live cells at lowest dilution ratio of 1:40 (1:40, 1:80, 1:120) during cryopreservation of *C. carpio*.

Effect of freezing rate on the milt quality

Motility duration

The highest initial mean post-thaw motility duration of 77.14± 4.38 s was observed when FP-I was used which decreased to 62.28± 2.12 s on 30th day (Table II). The reduction in motility duration was 15%. The lowest post-thaw motility duration was obtained when FP-III was used with initial value of 68.33 ± 1.63 s that declined drastically to 44.9±0.87 s (Table II) at the end of cryopreservation with reduction in the motility duration by 23%. The values obtained were statistically significant (p<0.001) when analysed using paired sample t test.

Table II. Motility duration and fertilizing ability of cryopreserved milt with three different freezing protocol at the end of experiment.

Freezing protocol	Motility duration (s)		Fertilization rate (%)	Hatching rate (%)
	Initial	Final		
I	77.14±4.38[a]	62.28±2.12[a]	77.3±1.63 [a]	61.6±2.44[a]
II	70.66±1.69[b]	59.33±1.28[b]	74.33±0.81[b]	59.6±2.16[b]
III	68.33±1.63[c]	44.9±0.87[c]	70.6±2.16 [c]	53.3±2.94[c]

Data expressed as Mean ± SE (n=5, r=3); Mean values in same column with different subscript differ significantly (p<0.0001). Paired sample t test was used in SPSS-22.0

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The freezing programme followed in FP-I had the slow rate of freezing (4°C/min) that had the best protective effects for cells during freezing when followed by rapid thawing as compared to the other two freezing rates (5°C/min and 10°C/min). The rate of cooling largely influences the success of cryopreservation as described by several authors (Lahnsteiner *et al*., 2003; Irawan *et al*., 2010) as it affects the post-thaw motility of spermatozoa by influencing the formation of ice crystals and osmotic pressure during the phase of freezing. However, highest post-thaw motility may be obtained from using a combination of two-step cooling rates (Conget *et al*.,1996; Suquet *et al*., 2000).

The present work falls in line with the work of Urbanyi *et al*. (1999), who reported successful use of slow freezing rate (4°C/min) during the cryopreservation of African catfish (*Clarias gariepinus)*. Linhart *et al*. (2000) followed a similar cooling program following slower cooling rate (4°C/min) during the cryopreservation of Bohemian common carp, which showed high post-thaw motility duration (60 s) as compared to other cooling rates. The results obtained from the present study also correspond with the works carried out by Cognie *et al.* (1989), Margary *et al*. (1996) and Sultana *et al*. (2009).

The possible explanation for better post-thaw motility at slow freezing rate may be that, the slow cooling leads to increased water permeability leading to equilibration by transfer of internal water to external ice thus maintaining the osmotic balance. However, due to decreased water permeability, rapid cooling produces intracellular crystals which gets enlarged during warming because of their high surface energies. Rapid cooling is more damaging than slow cooling for various other cells too (Mazur, 1970).

Different types of cells may require different cooling rates, which also depend on the nature of extender and

concentrations of cryoprotectants used (Suquet *et al*., 2000). In contrast to the present work, various researchers (Bernath *et al*., 2015; Boryshpolets *et al*., 2017) obtained high post thaw motility duration at higher cooling rates. Irawan *et al*. (2010) obtained highest post thaw motility duration (99.7±12.8 s) when the milt of *C. carpio* was cryopreserved at the rate of 10°C/min from an initial temperature of 25°C to −40°C and then free fall to −180°C.

Percentage of live and dead spermatozoa

The highest percentage of live cells of 84% was observed with FP-I which decreased to 62% on the last day of cryopreservation. When FP-II was followed, percentage of live cells recorded was 81% which dropped to 63% at the end of experiment. The lowest live cells of 77% was recorded when FP-III was employed which declined to 53% on 30th day.

The present work was in accordance with Cognie *et al*. (1989), who reported intact spermatozoa (66%) after thawing. Horokhovatsky *et al*. (2018) reported decline in live cells from 96% to 70% after cryopreservation of sterlet (*Acipenser ruthenus*). Conget *et al*. (1996) showed higher percentage (63%) of live spermatozoa and progressive motility during cryopreservation of *O. mykiss*. Linhart *et al*. (2005) reported 57.6% of intact spermatozoa after cryopreservation of Wels catfish (*Silurus glanis*).

Fertilizing ability

From Table II, it can be seen that the highest fertilisation and hatching rate of 77.3±1.63% and 61.6±2.44% was observed when milt frozen with FP-I was used. The milt samples frozen using FP-II yielded 74.33±0.81% of fertilisation rate and 59.6±2.16% of hatching rate (Table II). The lowest fertilisation and hatching rate of 70.6±2.16% and 53.3±2.94%, respectively was obtained when the milt was cryopreserved following FP-III (Table II). The values obtained were statistically significant ($p<0.001$) when analysed using paired sample t test.

The results comply with the reports of Irawan *et al*. (2010), who reported fertilisation and hatching rate of about 73.6±6.5 % and 62.8±5.9 %, respectively in *C. carpio* when slow rate of freezing was followed. Additionally, Warnecke and Pluta (2003) observed no difference in hatching rates (80 ±2 %) and swim up (78±2%) between cryopreserved milt and fresh milt of *C. carpio* when slow cooling was employed during cryopreservation. Furthermore, Vuthiphandchai *et al*. (2015) reported fertilisation and hatching rates of 64.5± 4.6% and 45.4±5.2%, respectively with cryopreserved milt of Silver barb (*Barbodes gonionotus*) which was almost similar to that of fresh milt.

Contrary to our results, Linhart *et al*. (2000) obtained lower fertilisation (56±10 %) and hatching rates (52±9%) in *C. carpio* though they yielded very high post-thaw motility duration of spermatozoa. The inconsistency among the studies may be attributed to the quality of spermatozoa post thawing and also the number of thawed spermatozoa used for fertilisation of the eggs (Lahnsteiner *et al*., 1996). Billard and Cosson (1992) stated that diminished post-thaw spermatozoa quality may be due to irregular sensitivity to the duration of storage. Correspondingly, the variability occurred in frozen-thawed spermatozoa due to individual and seasonal fluctuation of gamete quality may also influence the duration of motility and fertilisation success after cryopreservation (Lubzens *et al*., 1997; Linhart *et al*., 2000).

CONCLUSION

The present study demonstrated the superiority of non-induced fish as regards to spermatological properties over the induced fish. The study also concluded successful cryopreservation of *C. carpio* milt at 1:10 dilution ratio following slow freezing rate. However, the level of cryoinjuries the spermatozoa undergoes during various freezing rates to be studied to develop a standard freezing protocol.

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ACKNOWLEDGEMENTS

The ICAR merit fellowship is greatly acknowledged for the funding support throughout the PG programme.

Funding

There was no funding received for this study.

IRB approval and ethical statement

The experiment was conducted following the procedures of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Government of India on care and use of animals in scientific research.

Statement of conflict of interest

The authors have declared no conflict of interest.

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