

Optimisation of sperm cryopreservation protocol for Asian stinging catfish *Heteropneustes fossilis* (Bloch, 1794), to support hatchery seed production

Kuldeep K. Lal^{1*}, Anindya S. Barman², Peyush Punia¹, Praveen Khare¹, Vindhya Mohindra¹, Rajeev K. Singh¹, Rama S. Sah¹, Sullip K. Majhi¹ and Bechan Lal³

¹ICAR-National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkhusa P. O., Lucknow-226 002, Uttar Pradesh, India

²College of Fisheries, Central Agricultural University, Lembucharra, Agartala-799 210, Tripura, India

³Department of Zoology, Banaras Hindu University, Varanasi-221 002, Uttar Pradesh, India

Abstract

The present study experimentally evaluated different parameters such as extender composition, storage duration and egg to sperm ratio to optimise parameters for sperm cryopreservation in Asian stinging catfish *Heteropneustes fossilis*. Four extender compositions viz. Hanks Balanced Salt Solution (HBSS), Modified HBSS (M-HBSS), Modified HBSS with hen's egg yolk (M-HBSS with EY) and European catfish extender were evaluated for sperm cryopreservation with dimethyl sulphoxide (DMSO) as cryoprotectant (10% v/v). The hatching rate was used as standard to evaluate the fertility of thawed sperm at different periods of storage (2, 270 and 668 days). After 2 days, extender M-HBSS exhibited highest hatching rate (49.06±2.67%) which was comparable to the control value (51.00±7.23%), followed by HBSS (42.76±32.0%), M-HBSS with EY (37.46±24.34%) and European catfish extender (29.47±25.61%), respectively. After 270 days with M-HBSS, hatching rate was 64.34±8.84% similar to the control; however, cryopreserved sperm with other extenders exhibited lower hatching rate as compared to control and M-HBSS. After 668 days, sperm cryopreserved in the extender M-HBSS, only yielded successful hatching. Results indicated that presence of egg yolk in the extender M-HBSS did not improve the performance of cryopreserved sperm. The successful hatching using cryopreserved sperm after 270 and 668 days of storage indicates M-HBSS as a useful extender for sperm cryobanking and seed production of *H. fossilis*.



*Correspondence e-mail:

kuldeep.lal@icar.gov.in, kuldeepklal@gmail.com

Keywords:

Aquaculture, Cryopreservation, Cryoprotectant, Dimethyl sulphoxide, Extender, Hanks Balanced Salt Solution, Hatching rate

Received : 08.10.2020

Accepted : 20.06.2023

Introduction

Sperm cryopreservation is a useful tool for seed production of catfish species, because usually catfishes are sacrificed for sperm collection and milt quantity is very low. The cryo-sperm banks can play important role in future for generation of genetically diversified fish seed for aquaculture (Diwan *et al.*, 2020). Cryopreserved sperm can be used to improve breeding programmes in hatcheries through ready availability of milt at the time of need and hence, will help optimum usage of milt which has direct implication on aquaculture industry. It also has pivotal role in conservation of valuable genetic

resources. *Heteropneustes fossilis* (Bloch) (Order: Siluriformes; Family: Heteropneustidae) commonly known as Asian stinging catfish is an important air breathing fish in the South Asian countries, especially in the Indian subcontinent. This catfish species is hardy and omnivorous in nature, can tolerate poor water quality and cultured in derelict, swampy and marshy water areas. *H. fossilis* has high nutritive and pharmaceutical values with great consumer preference and high market price. Several anthropogenic activities like unsustainable fishing and habitat alterations have resulted in the threatened status of this species in nature (Haniffa *et al.*, 2008). *H. fossilis*

is reported to have 43.56 eggs per g body weight, however, absolute fecundity is low due to small body size of brooders i.e., body weight ranging from 100-300 g (Dasgupta and Sengupta, 2014). The captive breeding of this fish species is mostly achieved through surgical removal of testis and thus require sacrificing the male (Ramaswami and Sundararaj, 1956; Sundararaj and Goswami, 1966; Devaraj, et al, 1972), though non-invasive natural breeding has also been achieved (Saika et. al., 2022). Collectively these factors pose major constraints for inadequate seed availability, thus is a bottleneck in advancement of its aquaculture and need for sacrificing males poses limitation in the hatchery seed production. Under such situation, cryopreservation of sperm will help in the optimum use of sperm according to the quality eggs available for fertilisation. As a part of aquaculture diversification, *H. fossilis* is considered as a species of aquaculture interest for improving rural income (Roy et al., 2019), in combination with other species (Azhar et al., 2018) and in emerging new aquaculture systems such as re-circulatory aquaculture systems (RAS) and biofloc (Laxamappa, 2015). *H. fossilis* culture is a small-scale activity in south Asia. Availability of an easy-to-use sperm cryopreservation protocol, with simple and low-cost technologies, is likely to attract confidence of the seed producers.

The need for species-specific protocol for milt cryopreservation is met through experimental standardisation of various parameters. Successful methods for cryopreservation of catfish sperm have been reported for several species (Gwo, 2000; Chew and Zulkafli, 2012). DMSO is the most commonly used cryoprotectant for sperm cryopreservation in a number of fish species including catfishes (Urbanyi et al., 1999; Horvath and Urbanyi, 2000; Wayman and Tiersch, 2000; Kwantong and Bart, 2003; Basavaraja and Hegde, 2004; Lal et al., 2009; Diwan et al., 2010). In *Silurus glanis*, 82-86% hatching rate were obtained with sperm preserved 5 h before freezing in immobilising solution Me2SO (Linhart, et al, 2005). Christensen and Tiersch, (2005) compared the effects of freezing and thawing on motility of sperm in Channel catfish *Ictalurus punctatus*. In *Pangasianodon hypophthalmus*, maximum fertilisation (41%) was obtained with the combination of 12% DMSO and 0.9% NaCl (Kwantong and Bart, 2003). Urbanyi et al. (1999) reported highest fertilisation rate (96%) in *Clarias gariepinus* with either glucose or fructose as extender.

We previously reported the successful cryopreservation of *C. batrachus* and *H. fossilis* spermatozoa where in *H. fossilis*, extender M-HBSS resulted in highest hatching rate (49.06%) which was almost similar to control i.e., 51% and in *C. batrachus* HBSS showed highest hatching rate (62.1%), though it was found significantly low as compared to control (Lal et al., 2009). In our earlier study on this fish, Computer Assisted Sperm Assessment (CASA) was used to assess the parameters of sperm motility in fresh as well as cryopreserved samples, and in response to treatments (Barman et al., 2013). The study revealed positive role of nitric oxide in improving the fertility of normal and cryopreserved sperm in *H. fossilis*.

In this study, we report the experimental standardisation of protocol and parameters for *H. fossilis* sperm cryopreservation, with an aim to implement the technique in hatchery seed production. Here, we evaluated sperm cryopreserved with different extender compositions and stored for different durations. In this study, direct count of fertilised eggs and hatchability was used as index of performance of the protocols, which is relevant to hatchery seed production. The results reported here can be potentially used for upscaling of *H. fossilis* milt cryopreservation in order to address the problem of milt availability of this catfish in commercial seed production and aquaculture development.

Materials and methods

Ethical statement

This study was approved by the Animal Ethics Committee of ICAR-National Bureau of Fish Genetic Resources, Lucknow. All the fishes used in the experiments were handled according to the prescribed guidelines.

Collection of fish and acclimatisation

Adult *H. fossilis* with average body weight (BW) of 168.67 g (SE±3.76) and body length of 17.06 cm (SE±0.26) were obtained locally, four to five months prior to the breeding season (June to August) and acclimatised in captive conditions in 500 l fiber glass reinforced plastic (FRP) tanks, at ambient photoperiod (12L:12D) and water temperature (26°C). Fish were fed with chopped fish meat and commercially available pelleted prawn feed @3-5% of body weight. Aquatic weeds and hide-outs were provided in rearing tanks to simulate the natural habitat condition.

Selection of brooders and induced breeding

Mature male and female (15 Nos. each) fishes were selected based on sexual dimorphic characteristics (Chondar, 1999). The selected male and female brooders were kept in separate tanks for 3-4 days before experimentation. Synthetic hormonal formulation Ovaprim (Syndel Laboratories LTD, Canada.) was injected intramuscularly below the dorsal fin at the rate of 0.6 ml per kg body weight to females, and at 0.3 ml per kg body weight to males. After injection, male and females were maintained in separate flow-through tanks (water temperature: 26±2°C) with continuous shower.

Collection of sperm suspension

After the latency period of 14 h, males were sacrificed by anesthetic overdose (clove oil 0.50 ml l⁻¹ water) and testes were removed. Testicular tissue of male was cut

into small pieces using a sterile scissor and macerated in mortar and pestle with 0.9% NaCl solution (1: 3.5 g ml⁻¹) and content was sieved through bolting silk (0.22 µm). The sperm suspension was collected and centrifuged at 5000 rpm for 2 min to segregate debris. Then 200 µl of concentrated supernatant from the middle column, avoiding bottom debris, of the tube was collected from each suspension and was observed immediately under microscope (200x) to record the percentage of motile sperms. The milt samples exhibiting more than 70-80% sperm motility were pooled from 10 individuals and used for cryopreservation. A part of milt samples (0.05 ml) was fixed in eosin mixed with 10% neural buffered formalin (1.95 ml) for counting. The sperm concentration of pooled milt was determined by counting spermatozoa (1:200 dilution) in a haemocytometer counting chamber (Neubauer, Blankenburg, Germany).

Sperm cryopreservation

The protocol for sperm cryopreservation was as described by Lal et al. (2009). Briefly, after pooling of non-activated milt, sperm suspension was diluted with different extenders, used for the study, separately and cryoprotectant in the ratio 1:3.5:0.5 (milt: extender: cryoprotectant). Dimethyl sulphoxide (DMSO) was used as cryoprotectant at a concentration of 10% v/v with all the extenders. In the present study, four different extenders were evaluated for their suitability to cryopreserve the milt of *H. fossilis*. The sperm suspension, diluted with extenders and cryoprotectants, was filled in 0.25 ml French straws (Cryo BioSystems, India) and sealed with polyvinyl alcohol (PVA) powder. The straws were then placed over ice flakes for equilibration for 10 min followed by exposure to liquid nitrogen vapour by horizontal freezing over a steel mesh stand in a thermal insulated box (about 3 cm above the LN₂ surface, temperature about -70°C) for 10 min and then plunged in LN₂.

Evaluation of hatching ability of sperm

After the latency period of 14 h, eggs from individual female fish were collected by hand stripping after wiping the genital aperture with dry cloth. The brownish green-coloured transparent eggs were considered good quality and pooled for fertility evaluation. The fertility evaluation of sperm suspensions cryopreserved with four different extenders viz. Hanks Balanced Salt Solution (HBSS), Modified HBSS (M-HBSS), Modified HBSS with hen's egg yolk (2%) and European catfish extender as well as freshly obtained sperm suspension (control) was carried out in triplicates. For fertilisation test, 100 µl of egg (68±8) were drawn using micropipet (100-1000 µl; Nichiryo, Japan) attached with cut tips, were fertilised with one straw (0.25 ml). To thaw the sperm suspension, the straws were removed from LN₂ and immediately immersed in water bath at 37°C for 15 s and wiped dry. The thawed sperm suspension was directly poured over the eggs and mixed thoroughly. Fifty microliters of pond water (pH 7.4), filtered through Whatman (A1) filter paper was added as an activator to the egg-sperm mixture for 2-3 min followed by washing of mixture with well aerated pond water and incubation in 100 ml pond water for 15 min for water hardening. Water hardened embryos were transferred to flow through water tanks (26±2°C), group-wise separately, and incubated for 20-24 h for hatching. Assessment of embryonic development under a stereo zoom microscope was started 2 h after fertilisation and numbers of live embryos were scored. Number of hatchlings per group was counted after complete hatching.

Extender compositions and duration of storage

The four extender compositions (Table 1), HBSS, M-HBSS, M-HBSS with 2% hen's EY and European catfish extender were assessed for fertilisation and hatching rate of cryopreserved sperms after-storage duration of 48 h, 270 days and 668

Table 1. Composition of the four extenders used in the study

Constituent	HBSS	M-HBSS	M-HBSS with Egg yolk	European catfish
NaCl	137 mM	137 mM	137mM	200.15 mM
KCl	50.36 mM	50.36 mM	50.36 mM	-
CaCl ₂	1.26 mM	1.43 mM	1.43 mM	-
NaHCO ₃	0.35 mM	0.72 mM	0.72 mM	-
MgSO ₄	0.41 mM	0.41 mM	0.41 mM	-
NaH ₂ PO ₄	0.34 mM	0.68 mM	0.68 mM	-
Glucose	5.55 mM	5.55 mM	5.55 mM	-
KH ₂ PO ₄	0.44 mM	0.44 mM	0.44 mM	-
Tris	-	-	-	300 mM
Egg yolk	-	-	2%	-

days. The pooled sperm suspension yielded a total of 39 straws (0.25 ml) for 4 extenders and same were used in the fertilisation test after different periods of cryostorage. The pooling of milt helped to obtain adequate straws of same collection, for use in all the experiments which were conducted at different time intervals. Pooling from different males, which showed presence of motile sperms, was used as the method of choice for preparing adequate experimental milt in our previous work on *H. fossilis* (Lal *et al.*, 2009; Barman *et al.*, 2013); another small sised Asian catfish *Ompok pabda* (Rafiqul *et al.*, 2013) and in other species such as brown trout (Giusy *et al.*, 2019).

Sperm:egg ratio

Three sperm-egg ratios were tested in the present study viz. (i) 100 μ l eggs (68 ± 8) fertilised with one straw; (ii) 400 μ l of eggs (545 ± 5) fertilised with one straw and (iii) 400 μ l of eggs (545 ± 5) fertilised with two straws. The number of sperm per egg was calculated by dividing the quantity of sperm in the straw by the number of eggs inseminated. Number of eggs in 100 and 400 μ l were estimate based on actual count of three samples (Mean $\pm$ SD) for each volume. Hatching values under different treatments in each replicate was scored through the counting of hatchlings. For this experiment, a set of 32 straws (0.25 ml) was cryopreserved using milt from 10 males and M-HBSS extender with DMSO as cryoprotectant (as explained in previous sections)

Statistical analysis

Fertilisation and hatching rate are presented in terms of percent mean $\pm$ SD. Statistical analyses of data were carried out using SPSS (ver.16.0) software. Data were arcsine transformed to homogenise the variance. The transformed data was subjected to one way analysis of variance (ANOVA) supplemented with Leven's test and Duncan's multiple range test at 95% confidence limit.

Results

Fertility evaluation of sperm cryopreserved with different extenders and storage durations

For 48 h storage duration, highest fertilisation was observed with European catfish extender ($80.24 \pm 9.71\%$), followed by HBSS ($79.19 \pm 11.59\%$) and did not differ significantly ($p > 0.05$) with the control value ($81.33 \pm 15.12\%$). Fertilisation with M-HBSS ($67.63 \pm 7.64\%$) and M-HBSS with EY ($52.73 \pm 19.85\%$) were significantly lower than the control value. Maximum hatching rate was observed with extender M-HBSS ($49.06 \pm 2.67\%$), followed by HBSS ($42.76 \pm 32.0\%$), M-HBSS with EY ($37.46 \pm 24.34\%$) and

European catfish extender ($29.47 \pm 25.61\%$), respectively (Fig. 1 and 2). The hatching rate with sperm cryopreserved in M-HBSS extender was approximately 96% to the control value of $51 \pm 7.24\%$ and the two values did not differ significantly ($p > 0.05$).

Evaluation of sperm stored for 270 days showed highest fertilisation rate of $92.83 \pm 4.22\%$ for M-HBSS which was not significantly different ($p > 0.05$) from control ($95.00 \pm 2.21\%$), followed by M-HBSS with EY ($82.65 \pm 5.37\%$), European catfish extender ($69.56 \pm 3.24\%$) and HBSS ($68.5 \pm 4.22\%$). The extender M-HBSS also yielded highest hatching rate ($64.34 \pm 8.84\%$) approximating 91.1% of control value ($70.00 \pm 3.21\%$), followed by M-HBSS with EY, European catfish extender and HBSS $15.12 \pm 2.28\%$, $33.33 \pm 14.13\%$ and $17.38 \pm 2.28\%$, respectively (Fig. 1 and 2).

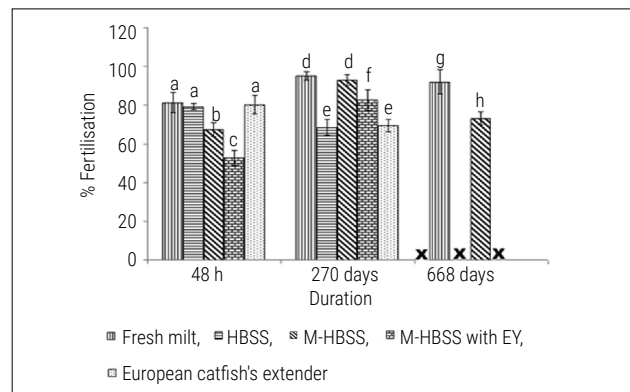


Fig. 1. Fertilisation (mean $\pm$ SD%) in *H. fossilis* obtained with freshly prepared sperm suspension (Control) and cryopreserved sperm with four different extender compositions (HBSS, M-HBSS, M-HBSS with EY, European catfish extender) stored for 48 h (Baseline Lal *et al.*, 2009) compared with 270 days and 668 days. The bars marked with different alphabets; indicate significant differences ($p < 0.05$).

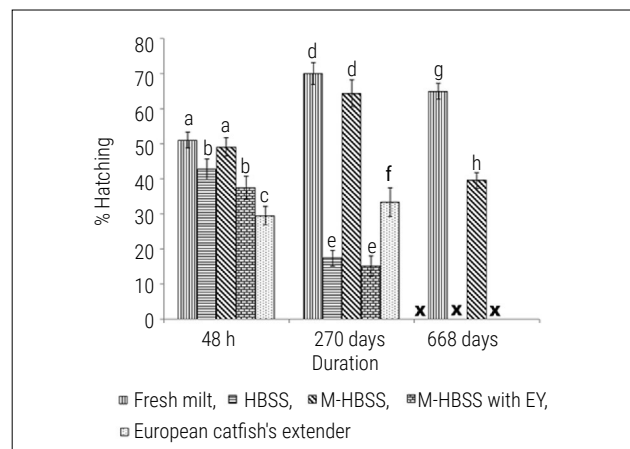


Fig. 2. Hatching (mean $\pm$ SD%) in *H. fossilis* obtained with freshly prepared sperm suspension (Control) and cryopreserved sperm with four different extender compositions (HBSS, M-HBSS, M-HBSS with EY, European catfish extender) stored for 48 h (Baseline Lal *et al.*, 2009) compared with 270 days and 668 days. Cross (X) represents no hatching. The bars marked with different alphabets; indicate significant differences ($p < 0.05$).

For fertility evaluation of sperm stored for 668 days, only M-HBSS exhibited successful fertilisation and hatching while the other three extenders failed to show viable hatching. M-HBSS exhibited $73.1 \pm 3.42\%$ fertilisation compared to control ($92.00 \pm 6.33\%$) and hatching of $39.55 \pm 8.44\%$, which was 61.33% of control value (Fig. 1 and 2). The results clearly showed that M-HBSS exhibited better performance for *H. fossilis* sperm cryopreservation compared to other extender compositions tested, however, hatching success at 668 days was only 61.33% of control value. The hatching yield from sperm cryopreserved with M-HBSS extender, after storage of 48 h (96% of control) and 270 days (91.1% of control) did not differ significantly ($p > 0.05$) from their respective control values. After 668 days of cryostorage, hatching yield with the same extender was significantly lower ($p < 0.05$) than control value (61.33% of the control value).

Mortality of embryos was affected by extender composition and storage duration (Fig. 3). Our result indicates that after 48 h of storage, M-HBSS with egg yolk showed lowest mortality percentage (15.27%), followed by HBSS (30.33%), M-HBSS (36.43%) and European catfish extender (50.77%). Similarly, after 270 days of storage, lowest mortality was observed with the extender M-HBSS (28.49%), followed by European catfish extender (36.23%), HBSS (51.12%) and M-HBSS with egg yolk (67.53%). No significant correlation between fertilisation and hatching value could be established from these fertility trials ($p > 0.05$).

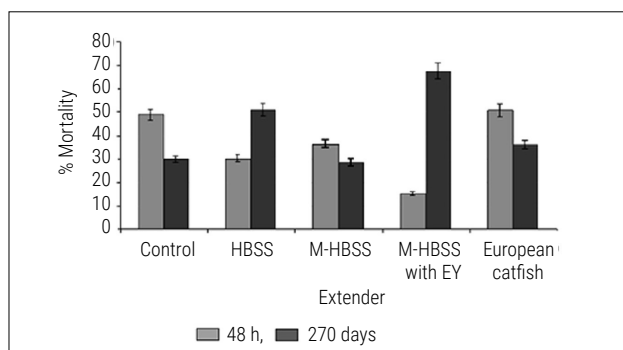


Fig. 3. Mortality (Mean $\pm$ SD%) of *H. fossilis* embryo between the fertilisation and hatchability counts of control and cryopreserved sperm with four different extender compositions and stored for 48 h and 270 days ($p < 0.05$).

Sperm:egg ratio

The mean sperm concentration in undiluted sperm suspension was $3.14 \times 10^9 \text{ ml}^{-1}$, which yielded concentration of 1.57×10^8 in 0.25 ml straws with diluted sperm suspension. The insemination ratios per egg was 2.31×10^6 in single 0.25 ml straw (treatment i, 100 μl egg) and 0.28×10^6 in one 0.25 ml straw (treatment ii, 400 μl egg). Comparison of hatching value for M-HBSS extender (48 h storage) obtained under different insemination ratios indicated that the hatching value (%) decreased significantly ($p < 0.05$) from $49.06 \pm 2.67\%$ (95.61% of control value) in treatment i to $10.33 \pm 3.2\%$

(20.13% of control value) in treatment ii. Similarly, hatchability enhanced significantly ($p < 0.05$) to $18.62 \pm 2.28\%$ (36.29% of control value) in treatment iii, with increase in sperm per egg (0.59×10^6) (Fig. 4).

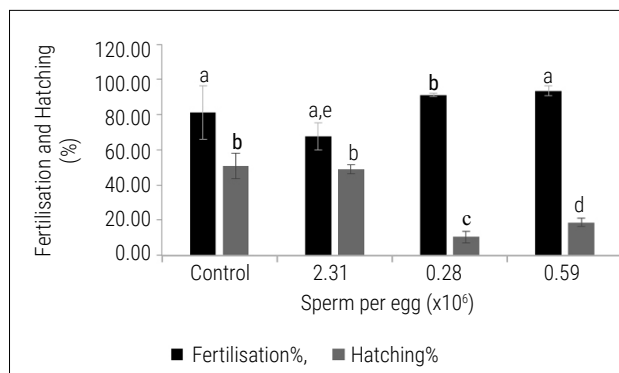


Fig. 4. Effect of sperm egg ratio on fertilisation and hatching of cryopreserved sperm of *H. fossilis*. The bars marked with different alphabets; fertilisation (a, e) and hatching (b, c, d), indicate significant differences ($p < 0.05$).

Discussion

All the four extender compositions used in the present study exhibited varying effects on *H. fossilis* sperm cryopreservation. Out of the four extenders used, M-HBSS out-performed other extenders with respect to maintaining the fertilising ability of cryopreserved sperm and subsequently hatching rate. Therefore, the extender M-HBSS proved to be suitable for sperm cryopreservation of *H. fossilis* at least for 1 to 2 years of storage. The extender compositions such as HBSS and M-HBSS with egg yolk (2% v/v) resulted in more than 3-fold decline in hatching during storage period of 270 days as compared to that observed in 48 days. Kim et al. (2020) described the reduction in viability of cryopreserved sperm during storage period of 5 months and 3 years in seven band groupers. The motility reduced from 90% in fresh to 61% after 5 months and 39% in 3 years. Similarly, number of surviving sperms reduced to 69% after 3 years of storage and revealed several cryo-damages during cryostorage. Ding et al. (2009) found similar performance of the sperm stored for 1 week and 1 year in *Siniperca chuatsi*. In *Pagrus major*, cryopreserved sperm stored up to 60 days exhibited fertilisation and hatching rate that was not different from the freshly derived sperm, which significantly declined after 1 year of cryostorage (Liu et al., 2010). In *Bryconor bignyanus*, motility and viability were found to be lower with cryo-stored sperm than fresh sperm (Maria et al., 2006). Very limited empirical data is available in literature to conclude how long the cryopreserved sperm can be viable under different protocols of storage, especially beyond 1 year. Nevertheless, this information could be important to make the technique dependable for aquaculture and conservation applications. Theoretically, under optimum cryopreservation and storage protocols, the viability of gametes can be preserved up to

32,000 years (Ashwood-Smith, 1980). In other words, the empirical data through experimentation is important to commit that a protocol is optimum and adequate for viable cryopreservation of the sperm for the extended period. The present results clearly demonstrated that the extender composition influence the duration of storage and all the extenders may not be suitable for long-term cryopreservation. The results suggested that evaluation of fertilisation and hatching rate of cryopreserved sperm at different duration of cryo-storage is necessary so that the protocol could be recommended as useful for a specified period of storage in seed production and germplasm storage programmes. This will enable an element of predictability in using sperm cryopreservation protocol for seed production

Poor correlation between fertilisation and hatching rate in the present data did not support the use of fertilisation rate (cell division) as an appropriate index of successful sperm cryopreservation protocol. Padhi and Mandal (1995) on sperm cryopreservation of the two catfish species *H. fossilis* and *C. batrachus* compared three different cryodiluents with glycerol as cryoprotectant; however, only up to a few cell divisions of embryonic development. Mortality observed in this study between intervening periods of fertilisation and hatching, points out that the survival of embryo after fertilisation is significantly different among the extender compositions. In other words, high fertilisation count did not ensure the corresponding hatching rate. Similar inference was drawn by Babiak and Glogowski (1998) on cryopreservation of asp sperm. Probably, under certain cryopreservation protocols, though sperms are motile and capable of fertilisation, might have suffered genomic damages rendering incapability to complete embryonic development (Li *et al.*, 2010). Our results clearly demonstrated that the effective cryopreservation protocols need to evaluate the production of viable hatchlings to ensure field adaptation of the technology. The count of early embryo development is not likely to be a reliable indicator of cryopreservation success and potentially, can also be confounded due to reported parthenogenetic development in some proportion of eggs (Siddique *et al.*, 2016). The hatching values, including control and M-HBSS extender, after 270 days of cryo-storage were considerably higher than the storage duration of 48 h and could possibly be attributed to the better egg quality during the second experiment.

Addition of egg yolk to the extender did not appear to improve the hatching rate in *H. fossilis*, rather it had negative impact. The hatching rate with M-HBSS with egg yolk was significantly lower than M-HBSS which further declined at 48 h storage and declined nearly three folds on 270 days of storage. The post-fertilisation mortality and hatching rate also declined with storage duration. Lowering of extender performance after addition of egg yolk has also been reported in *Labeo rohita* (Gupta and Rath, 1991); pike (Babiak *et al.*, 1995) and asp (Babiak and Glogowski, 1998). The hen's egg yolk is the most commonly used non-

permeating cryoprotectant (Rana, 1995). The lipoprotein fraction of egg yolk protects the membranes during freezing and improves fertilisation in mammalian sperm (Polge, 1980). The improved fertility with extender after addition of egg yolk (2-20%) has been reported in several fish species including rainbow trout (Baynes and Scott, 1987; Thakur *et al.*, 1997); brown trout (Piironen, 1993); pike (Babiak *et al.*, 1995) and common carp (Babiak *et al.*, 1997). The effect of egg yolk is opined to be synergistic with the constituents of extender and can have either positive or negative influence (Babiak *et al.*, 1995).

Egg to sperm ratio is an important feature in utilisation of sperm cryopreservation technology for commercial seed production. The hatchery seed production needs fertilisation of large volume of eggs. Hence, the optimum utilisation of gametes can contribute towards effectiveness of seed production using sperm cryopreservation approach. Here, we observed that the number of sperm per egg significantly affect the hatching success.

From our results, it is concluded that M-HBSS, with 10% DMSO as cryoprotectant can be used to cryopreserve *H. fossilis* sperm and such preserved milt can be recommended for use up to one year. The protocol has potential to produce viable hatchlings, approximately 50% of the number of eggs fertilised @ 2.31×10^6 sperms per egg. Extrapolating the results for a small-scale hatchery seed producer, by using a small cryocan (11 l capacity cryocan (IOC Ltd, <https://www.iocl.com/products/Cryocan.pdf>) that can hold over 1400 straws (0.5 ml) is adequate to fertilise over 190000 eggs and potentially will yield close to 100000 hatchlings. Further more, sperm cryopreservation can be used in combination with the recent reported non-invasive collection of sperm in catfish (Majhi *et al.*, 2020) which allows reuse of male brooder. This can be applied to *H. fossilis* and other catfishes for sperm collection, preservation and thus safe guarding precious broodstock of catfish species.

The present study optimised the protocol for sperm cryopreservation of *H. fossilis* and demonstrated its application in production of hatchlings which can be potentially used for hatchery production of seed of this catfish species. The results prove that the sperm can be effectively stored without loss of viability for close to one year and can be effectively used for hatchery purpose in next season. The simple tools and methods used in the study will encourage the semi-technical stakeholders for its adoption. The technology can help to alleviate the bottleneck in seed production of this commercially important species and promote aquaculture diversification and income of the small-scale rural farmers.

Acknowledgements

The authors thank the Director, ICAR-NBFGR, Lucknow for kind support during the implementation of the programme.

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