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Effect of Age on the Spermatological and Fertilisation Parameters of Common Carp *Cyprinus carpio* (Linnaeus 1758) Brooders Cryopreserved at Three Dilution Ratios

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Abstract

Cryopreservation is an important biotechnological tool that helps in producing quality fish gametes. Maintaining the quality of male and female gametes is essential since it may affect fertilisation success and larval survival. In the present study, the influence of age of common carp brooders on the spermatological and artificial parameters of cryopreserved milt was evaluated at three dilution ratios. Milt was collected from 6, 12 and 18 months old brooders of common carp, *Cyprinus carpio* (Linnaeus 1758) and diluted and cryopreserved at three dilution ratios viz. 1:40, 1:80 and 1:120 using freshwater fish saline as extender and dimethyl sulfoxide (DMSO) as cryoprotectant (90:10). Motility duration, fertilisation and hatching rates were studied for different treatments. Observations were made once in 15 days for 60 days after which artificial fertilisation was attempted. Brooders that were 12 months old exhibited the highest post-thaw motility duration (60.33±1.52 s), fertilisation rate (87.6±1.52 %) and hatching rate (57.3±4.1 %) when cryopreserved milt was used at 1:40 dilution ratio. The difference in the values between age group of brooders, dilution ratios and cryopreserved and fresh milt was statistically significant ($p<0.05$).

Keywords: *Cyprinus carpio*, dilution ratio, age, cryopreservation, spermatology

Introduction

During 2013, the production of *Cyprinus carpio* (Linnaeus 1758) has been reported to be 4,080,045 tonnes which has increased from 3,775,733 tonnes in 2012 (FAO, 2015). It is highly preferred as a food fish and is a commercially important fish species cultured worldwide. The demand for uninterrupted supply of fish seed can be met through cryopreservation of spermatozoa.

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Cryopreservation of animal gametes is considered a component in the strategy to save endangered species by facilitating the storage of their gametes in the gene bank (Gausen 1993). It is a tool for long term storage of spermatozoa (Cloud et al. 1990).

The quality of male and female gametes may affect fertilisation success and larval survival (Rurangwa et al. 2004). Generally, the sperm motility, sperm concentration, egg diameter, fertilisation rate and hatching rate were used as indices of gamete quality (Aliniya et al. 2013). Factors such as age, health status physiological condition, maturity stage etc. are known to play a role in the milt quality and reproductive performance of a species (Büyükhatipoglu and Holtz 1984; Trippel and Neilson 1992; and Vuthiphandchai and Zohar 1999). The spermatozoa motility and its duration have great influence on successful fertilisation (Babiak et al. 1999; Tekin et al. 2003). Higher motility duration was observed from males of 3 years old than in 2 years old common carp along with higher percentage of motile spermatozoa in 2 years old individuals (Aliniya *et al*. 2013). Decrease in motility duration was observed after cryopreservation in brown trout (Bozkurt et al. 2012). Fertilisation and hatching rate can be decreased after cryopreservation when compared to the fresh sperm (Honeyfield and Krise 2000).

Semen dilution is a process that has been carried out as a means to increase the number of eggs that can be fertilised with a small volume of semen (Billard 1985). In fish spermatozoa cryopreservation, dilution of the sperm fluid is one of the important steps that have been reported to improve fertilisation rates when compared with results obtained with undiluted milt (Poon and Johnson 1970; Rieniets and Millard 1987). Varied observations and reports could be found in relation to dilution ratio in *C. carpio* spermatozoa cryopreservation (Rana and McAndrew 1989; Gwo et al. 1991; Reenaselvi 1994; Lubzens et al. 1997; Lahnsteiner et al. 2003). An experiment was conducted to evaluate the effect of different dilution ratios such as 1:10, 1:20, 1:50, 1:100, 1:200, 1:300, 1:400 and 1:500 on sperm motility of zebrafish, *Danio rerio* (Hamilton, 1822) and higher motility duration was noticed when the milt samples were diluted at 1:10, 1:20 and 1:50, and motility decreased with increased dilution (Jing *et al.* 2009). However, a standardised dilution ratio is not specified for *C. carpio* spermatozoa to aid in effective artificial fertilisation after cryopreservation. Hence the aim of the present study was to evaluate the effect of different age group brooders on the spermatological and fertilisation parameters of cryopreserved spermatozoa of common carp with respect to three dilution ratios.

Materials and Methods

An earthen pond of size 0.1 hectare (50×20m) was used for the development of the broodstock and further rearing. The pond was stocked with 40 days-old *C. carpio* fingerlings. The fish were grown for a period of one and a half years. Matured fish were selected based on the identification features (Thomas et al. 2003). The selected males and females were stocked separately in net cages erected in the pond (Secer et al. 2004). The size of the cage was 5×3×3 cu ft. The matured *C. carpio* adults (ABW 137±80g) were stocked in low cost cages at a density of 1 kg per m^3 of water.

Milt was collected and processed from five brooders of *C. carpio* once they were 6 months old. The brooders were placed in a separate cage and the same brooders were used as donors of milt after 12 months and 18 months. The donor adults with mild oozing of milt were given hormonal inducement through WOVA-FH @ 0.5 ml/kg of body weight intramuscularly at the base of the dorsal fin during the early hours of the day. Milt collection was done by gentle stripping (Lubzens et al. 1997; Kurokura et al. 1984). The milt was collected in a sterile, pre-labelled 1.5 ml cryovial.

Spermatological parameters were analysed using a NIKON E360 microscope under phase contrast at 40x magnification. Suitable motility scores were also assigned (Betsy and Stephen 2014). Quality of the spermatozoa in the milt was evaluated through observation of their motility. To evaluate motility, about 10 µl of semen from each fish was placed on a glass microscope slide and 100 µl activation solution (0.3% NaCl) was added (Secer et al. 2004).

The milt was diluted with freshwater fish saline (NaCl 7.5 $g.L^{-1}$; KCl 0.2 $g.L^{-1}$; $NaHCO_3$ 0.2 $g.L^{-1}$; $CaCl_2$ 0.2 $g.L^{-1}$). Dimethyl sulfoxide (DMSO) was used as a cryoprotectant and the ratio of extender and cryoprotectant was 90:10. The collected milt was diluted at three dilution ratios such as 1:40, 1:80 and 1:120. No equilibration time was given (Stoss and Refstie 1983; Jamieson 1991; Babiak et al. 2001). The milt was then loaded into 0.5 ml IMV french straws and sealed with a polymer powder (IMV, France). Rapid freezing of straws was done for 20 minutes. In order to allow the straws to get uniform freezing effect, the distance between the straws and liquid nitrogen (LN_2) was maintained at 3 cm (Bozkurt et al. 2005). They were then transferred to cryocans (BA-11) containing LN_2. Observations were made once in 15 days for 60 days. During sampling, straws were thawed at 30 ˚C for 30 s in serological water bath (Bozkurt et al. 2005). The thawed milt was observed for spermatological parameters like motility duration and motility score. After 60 days of storage, the milt was tested for its efficacy in artificial fertilisation.

The dry method of *in vitro* fertilisation was practised (Tekin et al. 2003; Sultana et al. 2010; Aliniya et al. 2013). Fresh milt and eggs were collected from fish of 6, 12 and 18 months age and fertilised with fresh and cryopreserved milt which was diluted with freshwater fish saline at dilution ratios of 1:40, 1:80 and 1:120. Eggs from a single female were collected and divided into six batches with each batch containing approximately 500 fresh milt samples. The sperm-egg ratio of approximately 250,000 sperm/egg (Aliniya et al. 2013) was followed for both fresh and cryopreserved sperm. The fertilisation rate and hatching rate was calculated as follows:

$$\text{Fertilisation rate} = \left\{ \frac{\text{Number of fertilised eggs}}{\text{Total eggs}} \right\} \times 100$$ (Brommage and Cumaranatunga 1988)

$$\text{Hatching rate} = \left\{ \frac{\text{Number of healthy fertilised eggs}}{\text{Number of fertilised eggs}} \right\} \times 100$$ (Hanjavanit et al. 2008)

All the observations were processed and tabulated. The data were statistically analysed by SPSS 17.0. To determine whether there was any difference among the means, one-way analysis of variance (ANOVA) and the Duncan multiple range test were applied to the result and p values less than 0.05 were regarded as significant.

Results

When milt was collected from 6 months old *C. carpio* brooders, it had the highest mean post-thaw motility duration of 42.33±3.05 s on the 60th day of observation at 1:40 dilution ratio (Table 1). However, the lowest post-thaw motility duration noted in milt from 6 months old brooders was 32.66±1.52 s at a dilution ratio of 1:80.

The highest initial mean motility duration of 69.66±4.72 s was observed in milt sample collected from 12 months old brooders at 1:40 dilution ratio which decreased to 60.33±1.52 s at the end of 60 days as can be seen from Table 2. When milt was collected from 12 months old brooders, the lowest mean post-thaw motility duration observed was 46.33±2.08 s in 1:120 dilution ratio.

Brooders of age 18 months showed the highest initial mean motility duration of 59±4 s which on the 60th day of observation reached a post-thaw motility duration of 37.33±1.52 s (1:40 dilution ratio) (Table 3). It can be seen that, the lowest post-thaw motility duration exhibited by 18 months old brooders was 28±2.64 s at dilution ratio of 1:120. There were significant differences in the motility duration between the three age groups of brooders and dilution ratios ($p<0.05$).

Table 1. Observations on the motility duration(s) of milt collected from 6 months old *C. carpio* and cryopreserved with freshwater fish saline at three dilution ratios

Dilution ratios	Days of cryopreservation				
	1	**15**	**30**	**45**	**60**
1:40	61.66±1.52^{c}	59±6.08^{c}	50±3.46^{b}	42.33±1.52^{a}	42.33±3.05^{a}
1:80	60.33±1.15^{d}	55.6±2.51^{c}	41.33±1.52^{b}	35.33±1.52^{a}	32.66±1.52^{a}
1:120	57±1^{c}	43.6±4.72^{b}	38.33±2.08^{a}	36±3^{a}	34.33±1.52^{a}

Superscripts denote significant differences between treatments ($p<0.05$)

Table 2. Observations on the motility duration(s) of milt collected from 12 months old *C. carpio* and cryopreserved with freshwater fish saline at three dilution ratios

Dilution ratios	Days of cryopreservation				
	1	**15**	**30**	**45**	**60**
1:40	69.66±4.72^{b}	64.66±3.05^{a,b}	63.66±4.5^{a,b}	62.66±2.51^{a}	60.33±1.52^{a}
1:80	66.66±3.78^{b}	57±3^{a}	61.33±6.65^{a,b}	60.66±1.15^{a,b}	56.33±3.51^{a}
1:120	53±5^{b}	49.33±2.51^{a}	47±1.73^{a}	46±1^{a}	46.33±2.08^{a}

Superscripts denote significant differences between treatments ($p<0.05$)

Table 3. Observations on the motility duration(s) of milt collected from 18 months old *C. carpio* and cryopreserved with freshwater fish saline at three dilution ratios

Dilution ratios	Days of cryopreservation				
	1	15	30	45	60
1:40	59±4^{c}	57±3^{c}	48.33±2.08^{b}	44.33±0.57^{b}	37.33±1.52^{a}
1:80	54.33±3.05^{c}	53.33±2.51^{c}	50±7.93^{b,c}	42.33±3.51^{a,b}	35.33±4.50^{a}
1:120	56.66±2.51^{d}	53.33±2.51^{d}	42.66±2.51^{c}	37±3.60^{b}	28±2.64^{a}

Superscripts denote significant differences between treatments ($p<0.05$)

The milt collected and cryopreserved from 12 months old brooders alone showed the highest motility score of 8 at the end of 60 days, whereas milt samples collected from 6 months and 18 months old brooders exhibited a motility score of 7. The motility pattern of milt collected from all three age group brooders exhibited only forward movement even on the 60th day of observation.

The difference in the post-thaw motility duration and motility percentage is depicted in Fig. 1 and 2. When both the figures are compared, it can be inferred that the motility was higher in milt collected from 12 months old brooders and cryopreserved with freshwater fish saline at 1:40 dilution ratio.

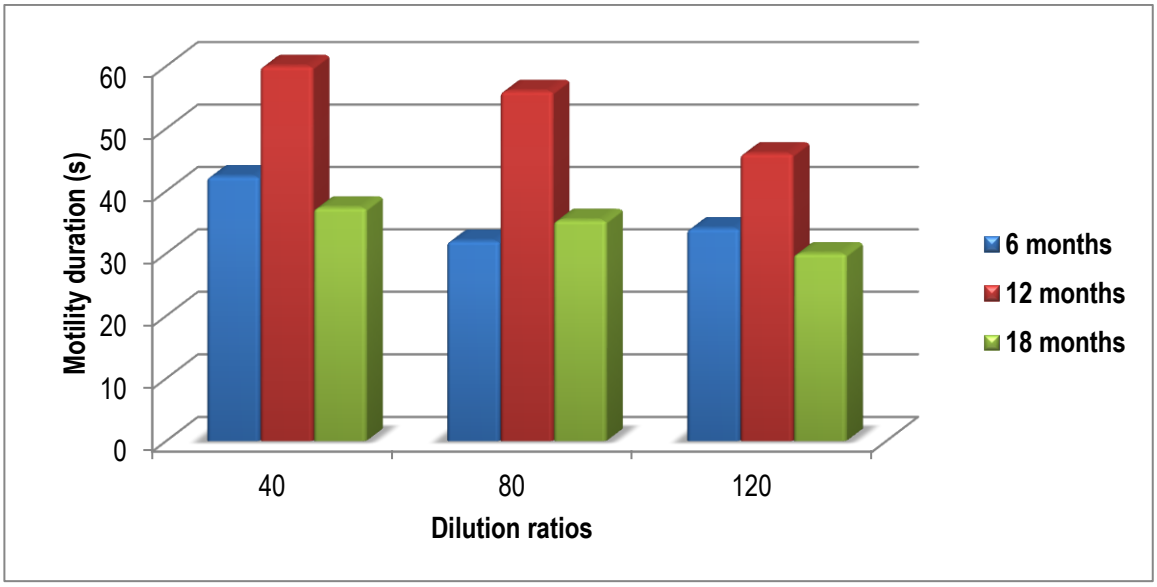

Fig. 1. Effect of dilution ratio on post-thaw motility duration(s) of *C. carpio* milt collected from three different age group brooders and cryopreserved with freshwater fish saline

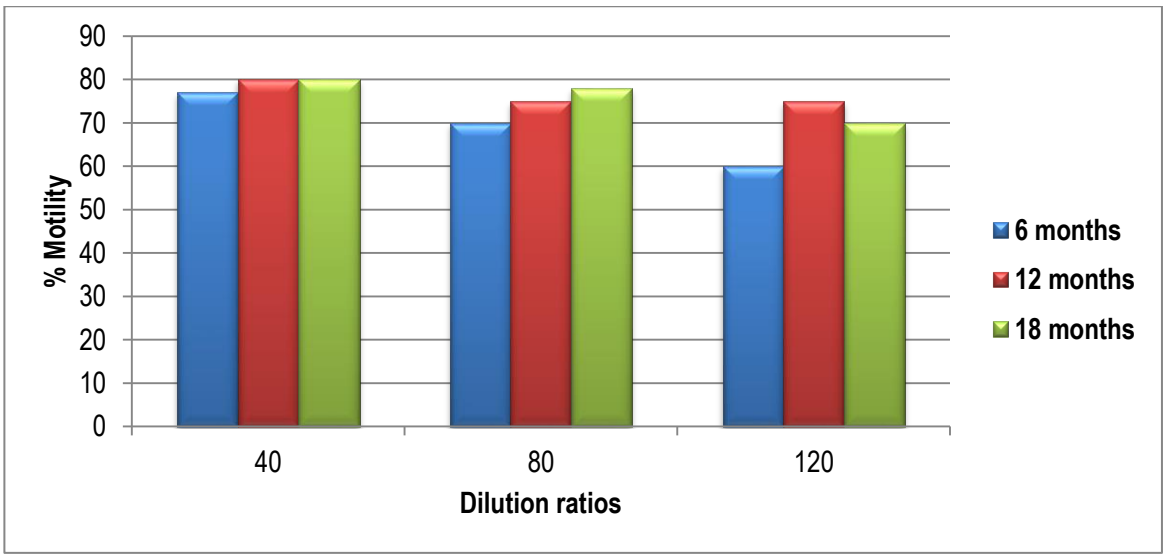

Fig. 2. Effect of dilution ratio on post-thaw motility percentage (%) of *C. carpio* milt collected from three different age group brooders and cryopreserved with freshwater fish saline

When gametes were collected from 6 months old brooders, maximum fertilisation rate of 76.3±0.57 % was obtained using cryopreserved milt and 79.3±2.08 % with fresh milt (Table 4) when milt diluted to 1:40 ratio was used. The lowest fertilisation rate observed was 66.6±2.51 % with cryopreserved milt and 69.3±2.51 % with fresh milt at 1:120 dilution ratio.

Higher fertilisation rate observed in 12 months old brooder was 87.6±1.52 % (cryopreserved milt) and 91.3±1.52 % (fresh milt) at 1:40 ratio. The lowest fertilisation rate obtained from 12 months old brooders with cryopreserved milt was 76.3±2.51 % and with fresh milt was 86.6±2.51 % (1:120 ratio).

The highest fertilisation rate obtained from 18 months old brooders using cryopreserved and fresh milt was 68.3±1.52 % and 72.3±2.51 % respectively when milt was diluted at 1:40 dilution ratio. Similarly, the lowest fertilisation rate of 67.6±3.05 % was noticed with fresh milt and 57.6±2.51 % with cryopreserved milt at 1:120 ratio. The fertilisation rate obtained using cryopreserved and fresh milt was statistically significant ($p<0.05$) between different age group brooders and dilution ratios.

From Table 5, it can be seen that the hatching rate of eggs fertilised with cryopreserved milt and fresh milt also followed the same trend as that of fertilisation rate. When cryopreserved milt was used for fertilisation, hatching rate obtained was very low when compared with fresh milt. The hatching rate was statistically significant ($p<0.05$) between different ages, dilution ratios and between cryopreserved and fresh milt.

Table 4. Fertilisation rate (%) obtained from eggs fertilised with fresh milt (FM) and cryopreserved milt (CM)

Dilution	6 months		12 months		18 months	
ratio	FM	CM	FM	CM	FM	CM
1:40	79.3±2.08	76.3±0.57	91.3±1.52*	87.6±1.52*	72.3±2.51	68.3±1.52
1:80	75.6±2.08	69.6±3.05	89.3±2.51	82.3±2.08	68.6±2.08	62.3±2.08
1:120	69.3±2.51	66.6±2.51	86.6±2.51	76.3±2.51	67.6±3.05	57.6±2.51

*($p<0.05$)

Table 5. Hatching rate (%) of eggs fertilised with fresh milt (FM) and cryopreserved milt (CM)

Dilution	6 months		12 months		18 months	
ratio	FM	CM	FM	CM	FM	CM
1:40	54.3±2.5	38.3±3.5	76.6±3.05*	57.3±4.1*	68.6±2.51	43.3±2.51
1:80	61.3±1.5	39.3±3.5	73.6±2.5	55.3±2.5	62.3±2.51	41.66±2.51
1:120	49.6±2	35.33±2.5	68.3±2.51	50.6±2.08	57.3±4.16	38.6±2.51

*($p<0.05$)

Discussion

Milt collected from 12 months old brooders alone maintained highest motility during and after cryopreservation (Table 2). Here the initial motility duration was 69.66±4.72 s which was reduced to the post-thaw motility duration of only 60.33±1.52 s. Brooders of 6 and 18 months age could not maintain the highest post-thaw motility and the values were statistically significant ($p<0.05$).

The reason for this might be the age of the brooders (Vuthiphandchai and Zohar 1999). It must be noted that aged gametes have their own limitation for cryopreservation (Billard 1986). The ability of spermatozoa to tolerate the stress of freezing and thawing may be altered during the course of spawning (Legendre and Billard 1980). Hence all these factors contributed to reduction in motility duration between age group brooders. From the results, it could also be said that milt diluted at 1:40 ratio and cryopreserved alone maintained the highest post-thaw motility of spermatozoa. This is because at higher cell concentrations, the post-thaw survival of sperm significantly decreases, which is attributed to cell compression because of limited intracellular space (Sarder et al. 2011). In support of this, there is a report stating that motility decreases with increasing dilution (Jing et al. 2009).

There was a difference in the fertilisation and hatching percentage between age groups of brooders which might be due to the poor quality of milt from 6 and 18 months old brooders which is directly reflected in low fertilisation and hatching rates as discussed by various researchers (Methven and Crim 1991; Shangguan and Crim 1995; Suquet et al. 1998). In the present study, the fertilisation rate decreased with increasing dilution ratios both in fresh and cryopreserved milt (Table 4).

This was corroborated by the fact that 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 milt dilution ratio had a strong effect on post-thaw motility and fertility (Rana and McAndrew 1989; Gwo et al. 1991). There was statistically significant difference ($p<0.05$) between the fertilisation rates of three dilution ratios.

The higher hatching rate of cryopreserved milt diluted at 1:40 ratio was 57.3±4.1 % and that of fresh milt was 76.6±3.05 %. When dilution ratio of 1:80 was used, the highest percentage of hatching rate noticed with cryopreserved milt was 55.3±2.5 % and with fresh milt it was 73.6±2.5 %. When milt was diluted at a ratio of 1:120, the higher hatching rate observed with cryopreserved milt was 50.6±2.08 % and 68.3±2.51 % for fresh milt (Table 5). All these maximum values were obtained from 12 months old brooders. The hatching rate obtained from different age group brooders with fresh and cryopreserved sperm at different dilution ratios alone was statistically significant ($p<0.05$). The fertilisation rate of cryopreserved spermatozoa was similar to that of fresh sperm although differences were observed in the hatching rate (Lahnsteiner et al. 2003). A reason for low hatching rate from cryopreserved spermatozoa might be due to the cryoinjury that could occur during freezing and thawing of the spermatozoa (Muchlisin 2004).

Conclusion

It can be inferred that milt collected and cryopreserved from 12 months old brooders at 1:40 dilution ratio alone exhibited the highest motility duration, fertilisation and hatching rates. As the age and dilution ratio increases, the milt quality decreases resulting in poor quality larvae.

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References

Aliniya, M., H. Khara, S.B. Noveiri and H. Dadras. 2013. Influence of age of Common Carp (*Cyprinus carpio*) brood stock on reproductive traits and fertilization. Turkish Journal of Fisheries and Aquatic Sciences 13: 19–25.

Babiak, I., L. Fraser, S. Dobosz, K. Goryczko, H. Kuzminski and J. Strzezek. 1999. Computer-controlled freezing of rainbow trout *Oncorhynchus mykiss* (Walbaum) spermatozoa for routine programmes. Aquaculture Research 30: 707–710.

Babiak, I., J. Glogowski, K. Goryczko, S. Dobosz, H. Kuzminski, J. Strzezek and W. Demianowicz. 2001. Effect of extender composition and equilibration time on fertilisation ability and enzymatic activity of rainbow trout cryopreserved spermatozoa. Theriogenology 56: 177–192.

Betsy, C.J. and J.S.K. Stephen. 2014. New classification of motility score in fishes to determine the quality of spermatozoa. International Journal of Fisheries and Aquatic Studies 1(4): 20–13.

Billard, R. 1985. Artificial insemination in salmonids. In: Salmonid Reproduction (eds. R.N. Iwamotoa and S. Sewer), pp. 116–118. Washington Sea Grants publication, Seattle.

Billard, R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reproduction, Nutrition, Development 26: 877–920.

Bozkurt, Y., E. Akcay, N. Tekin and S. Secer. 2005. Effect of freezing techniques, extenders and cryoprotectants on the fertilization rate of frozen rainbow trout *(Oncorhynchus mykiss)* sperm. Israeli Journal of Aquaculture– Bamidgeh 57: 125–130.

Bozkurt, Y., I. Yavas and F. Karaca. 2012. Cryopreservation of Brown Trout (*Salmo trutta macrostigma*) and Ornamental Koi Carp (*Cyprinus carpio*) Sperm. In: Current Frontiers in Cryopreservation (ed. I. Katkov). ISBN: 978-953-51-0302-8, InTech.

Bromage, N. and R.C. Cumaranatunga. 1988. Egg Production in the Rainbow Trout. In: Recent Advances in Aquaculture (eds. J.F. Muir and R.J. Roberts), pp. 63–138. Croom Helm, London.

Büyükhatipoglu, S. and W. Holtz. 1984. Sperm output in rainbow trout *(Salmo gairdneri)* effect of age, timing and frequency of stripping and presence of females. Aquaculture 37: 63–71.

Cloud, J.G., W.H. Miller and M.J. Levanduski. 1990. Cryopreservation of sperm as a means to store salmonid germplasm and to transfer genes from wild fish to hatchery populations. Progressive Fish Culturist 52: 51–53.

Cognie, F., R. Billard and N.H. Chao. 1989. Freezing of the milt of the common carp, *Cyprinus carpio*. Journal of Applied Ichthyology 5: 165–176.

FAO. 2015. Fishery and Aquaculture Statistics. Food and Agriculture Organization of the United Nations, Rome.

Gausen, D. 1993. The Norwegian gene bank programme for Atlantic salmon (*Salmo salar*). In: Genetic Conservation of salmonid fishes (eds. J.G. Cloud and G.H. Thorgaard), pp. 181–187, Plenum, New York.

Gwo, J.C., K. Strawn, M.J. Longnecker and C.R. Arnold. 1991. Cryopreservation of Atlantic croaker spermatozoa. Aquaculture 94: 355–375.

Hanjavanit, C., N. Kitancharoen and C. Rakmanee. 2008. Experimental Infection of Aquatic Fungi on Eggs of African Catfish (*Clarias gariepinus* Burch). KKU Science, 36: 36–43.

Honeyfield, D.C. and W.F. Krise. 2000. Measurement of milt quality and factors affecting viability of fish spermatozoa. In: Cryopreservation in Aquatic Species (eds. T.R. Tiersch and P.M. Mazik), pp. 49–58, World Aquaculture Society, Baton Rouge, Louisiana, USA.

Jamieson, G.M. 1991. Fish evolution and systematics: Evidence from spermatozoa. Cambridge University Press, Cambridge, pp. 319.

Jing, R., C. Huang, C. Bai, R. Tanguay and Q. Dong. 2009. Optimization of activation, collection, dilution and storage methods for zebrafish sperm. Aquaculture 290: 165–171.

Kurokura, H., R. Hirano, M. Tomita and M. Iwahashi. 1984. Cryopreservation of carp sperm. Aquaculture 37: 267–273.

Lahnsteiner, F., B. Berger and T. Weismann. 2003. Effects of media, fertilization technique, extender straw volume and sperm to egg ratio on hatchability of cyprinid embryos, using cryopreserved semen. Theriogenology 60: 829–841.

Legendre, M. and R. Billard. 1980. Cryopreservation of rainbow trout sperm by deep-freezing. Reproduction, Nutrition, Development 20: 1859–1868.

Lubzens, E., N. Daube, I. Pekarskay, Y. Magnus, A. Cohen, F. Yuesefovich and P. Feigin. 1997. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks - strategies in research and application. Aquaculture 155: 13–30.

Methven, D.A. and L.W. Crim. 1991. Seasonal changes in spermatocrit, plasma sex steroids and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*). In: Proceedings of the 4th International Symposium on Reproductive Physiology of Fish (eds. A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe), p. 170. Sheffield.

Muchlisin, Z.A. 2004. Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. Biodiversitas 6: 66–69.

Poon, D.C. and A.K. Johnson. 1970. The effect of delayed fertilization on transported salmon eggs. Progressive Fish Culturist 32: 81–84.

Rana, K.J. and B.J. McAndrew. 1989. The viability of cryopreserved tilapia spermatozoa. Aquaculture 76: 335–345.

Reenaselvi, 1994. Cryopreservation of carp spermatozoa. Ph. D. Thesis, Tamil Nadu Veterinary and Animal Sciences University. 166 p.

Rieniets, J.P. and J.L. Millard. 1987. Use of saline solutions to improve fertilization of northern pike eggs. Progressive Fish Culturist 48: 117–119.

Rurangwa, E., D.E. Kime, F. Olevier and J.P Nash. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234: 1–28.

Sarder, M.R.I., A.K.M.A. Rahman, M.S. Samad, K.M.S. Nazrul and M.K.J. Bhuiyan. 2011. Cryopreservation of sperm of *Labeo rohita* (Hamilton 1822) and its use in fertilization and hatching of eggs. Progressive Agriculturist 22: 123–137.

Secer, S., N. Tekin, Y. Bozkurt, N. Bukan and E. Akcay. 2004. Correlation between biochemical and spermatological parameters in rainbow trout *(Oncorhynchus mykiss)* semen. Israeli Journal of Aquaculture 56: 274–280.

Shangguan, B. and L.W. Crim. 1995. The effect of stripping frequency on the sperm quantity and quality in winter flounder, *Pleuronectes americanus*. In: (eds. F.W. Goetz and P. Thomas), p. 142. Proceedings of the Fifth International Symposium on Reproductive Physiology of Fish. Austin.

Stoss, J. and T. Refstie. 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and Sea Trout. Aquaculture 30: 229–236.

Sultana, M., M. Nahiduzzaman, M.M. Hassan, M.U.H. Khanam and M.A.R. Hossain. 2010. Fertility of cryopreserved common carp (*Cyprinus carpio*) spermatozoa. University Journal of Zoology Rajshahi University 28: 51–55.

Suquet, M., C. Dreanno, G. Dorange, Y. Normant, L. Quemener, J.L. Gaignon and R. Billard. 1998. The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization and storage capacities. Journal of Fish Biology 52: 31–41.

Tekin, N., S. Secer, E. Akcay, Y. Bozkurt and S. Kayam. 2003. The effect of age on spermatological properties in rainbow trout (*Oncorhynchus mykiss* W. 1792). Turkish Journal of Veterinary and Animal Sciences 27: 37–44.

Thomas, P.C., S. Ch. Rath and K.D. Mohapatra. 2003. Breeding and seed production of fin fish and shell fish. Daya Publishing House, Delhi. 402 pp.

Trippel, E.A. and J.D. Neilson. 1992. Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (*Gadus morhua*) and associated hatching success. Canadian Journal of Fisheries and Aquatic Science 49: 2118–2127.

Vuthiphandchai, V. and Y. Zohar. 1999. Age-related sperm quality of captive striped bass *Morone saxatilis*. Journal of World Aquaculture Society 30: 65–72.

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