



Captive broodstock development of common carp *Cyprinus carpio* Linnaeus, 1758: influence of age on spermatogenesis and fertilisation parameters

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ABSTRACT

Knowledge on the quality of male and female gametes which may affect fertilisation success and larval survival is necessary to enhance fish production to meet global demand. Gamete quality is affected by factors like sex ratio, stocking density, age, size, nutrition and feeding regime. In the present study, the spermatological and fertilisation parameters as influenced by the age of brooders of *Cyprinus carpio* Linnaeus 1758 developed under captive condition from a single population were studied with special reference to dilution ratios. Milt was collected from 6, 12 and 18 months old brooders of *C. carpio* and diluted at three ratios viz., 1:40, 1:80 and 1:120 using freshwater fish saline. Sperm density, spermatocrit value, seminal plasma composition, motility duration, fecundity, fertilisation and hatching rates were studied for brooders of all age groups. The results showed that milt from 12 months old brooder produced good quality milt in terms of motility duration (69.66 ± 4.72 sec), fertilisation rate ($91.3 \pm 3.05\%$) and hatching rate ($76.5 \pm 2.64\%$) at 1:40 dilution ratio.

Keywords: Age, Common carp, Dilution ratio, Fertilisation, Spermatogenesis

Introduction

Common carp *Cyprinus carpio* Linnaeus, 1758 is a highly preferred food fish and cultured worldwide. During 2013, the production of *C. carpio* was 4 million t (FAO, 2015) and ranked third globally. Common carp spawns throughout the year in tropical areas, with peaks in January-March and July-August. In wild condition, carps are partial spawners whereas domesticated carps release all their mature eggs within a few hours.

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 are used as indices of gamete quality (Aliniya *et al.*, 2013). Motility parameters are used to evaluate the quality of the spermatozoa (Billard *et al.*, 1995). Spermatozoa motility, milt volume and sperm concentration are considered as good indicators of milt quality (Cabrita *et al.*, 2001; Tekin *et al.*, 2003). The quality of fish eggs is generally determined by their fertilisation rate and hatching survival. Good quality eggs exhibit low mortality levels

at fertilisation, hatching and first feeding (Brooks *et al.*, 1997). Poor quality eggs have delayed cortical reaction and slow rise in osmolarity after fertilisation (Kjorsvik and Lonning, 1983).

The age of broodstock influences the quality of the gametes produced. Although there are no strong evidences or reports on the changes in the quantity of spermatozoa at different age, many researchers reported decreased relative fecundity in female fishes as age advances (Siraj *et al.*, 1983; Springate *et al.*, 1984). The age of broodstock also affects the success of sperm storage. According to Tempero *et al.* (2006), age at maturity in common carp is related to latitude and sex. They also stated that males mature before females and fish mature earlier at low latitudes compared to higher latitudes. Parameswaran *et al.* (1972) reported that in pond culture in India, males mature at 6 months of age and females at 8 months.

Considerable variations occur in the spermatological quality of milt collected from one individual at different times of the same season. Factors such as age, health status, physiological condition and maturity stage are known to play a role in the milt quality and reproductive

performance of a species (Buyukhatipoglu and Holtz, 1984; Trippel and Neilson, 1992; Vuthiphandchai and Zohar, 1999). The present study aimed at evaluating the effect of different age group of brooders on the spermatological and fertilisation parameters to identify the right age group for breeding.

Materials and methods

Seed stocking and broodstock development

Earthen pond of 0.1 ha (50 × 20 m) was used for the broodstock development and rearing. The pond was stocked with *C. carpio* fingerlings of 40 days age (5±1.3 g). The fishes were grown for a period of one and a half years. Mature fishes were selected based on the identification features described by Thomas *et al.* (2003). The selected 20 males and 20 females were stocked separately in low cost net cages (5×3×3 ft) erected in the pond at a density of 1 kg per m³ of water, following Secer *et al.* (2004).

Milt collection

Milt was collected and processed from 5 brooders of *C. carpio* once they reached 6 months age (137.07±2.58 g; 17.28±1.22 cm). Subsequently, the same brooders were used as donors for milt after 12 months (319.28±20.17 g and 26.24±2.65 cm) and 18 months (507.85±27.3 g and 30.53±1.75 cm). The donor adults with oozing milt on mild pressing of abdomen were given hormonal inducement using WOVA-FH @ 0.5 ml kg⁻¹ of body weight intramuscularly at the base of the dorsal fin during early hours of the day. Milt collection was done by gentle stripping as described by Kurokura *et al.* (1984) and Lubzens *et al.* (1997).

Spermatological parameters of milt

The volume of milt (ml) was directly determined from the graduations in the cryovials following Aral *et al.* (2007). Colour of milt was determined by visual observation. Samples of 100 µl were drawn from fresh milt for evaluation of pH using pH indicator strips (pH: 6.5-10; Merck, Germany) (Akçay *et al.*, 2004; Bozkurt *et al.*, 2012).

The collected milt was analysed under NIKON E360 microscope using phase contrast objective for estimating sperm density and motility duration. In order to estimate these parameters, milt was diluted with freshwater fish saline (FFS) at dilution ratios of 1:40, 1:80 and 1:120. Suitable motility scores were also assigned following Betsy and Stephen (2014). Sperm density was estimated using haemocytometer (NAUBAEUR, Germany) as described by Hafez (1987). Quality of the spermatozoa in the milt was evaluated by observation of their motility by placing 1µl of diluted milt sample and 1µl of tap water on

glass slide and observing under microscope (Secer *et al.*, 2004). Spermatocrit value was estimated following Verma *et al.* (2009) and expressed as percentage.

The seminal plasma collected after centrifugation of the milt at 1000 g for 10 min (Secer *et al.*, 2004). were used for estimation of magnesium and calcium content in Spectroquant NOVA 60 (Merck, Germany) using Photometric cell test kits (Merck, Germany).

Artificial fertilisation

Dry method of *in vitro* fertilisation was employed following Sultana *et al.* (2010) and Aliniya *et al.* (2013). Fresh milt was collected from the fishes of 6, 12 and 18 months age groups and fertilised with the fresh milt which was diluted with FFS at dilution ratios of 1:40, 1:80 and 1:120. Eggs from single female was collected and divided into 3 batches and each batch contained approximately 500 eggs. Batches of eggs were inseminated with fresh milt samples. The sperm-egg ratio was approximately 250000 sperm per egg (Aliniya *et al.*, 2013). Fertilisation was done in dry plastic dishes. The fertilisation rate and hatching rates were calculated following Bromage and Cumaratunga (1988) and Hanjavanit *et al.* (2008) respectively.

Statistical analysis

The data were statistically analysed by ANOVA in SPSS 20 software.

Results

The milt was milky white in appearance and the pH varied from 7-8. Volume of milt collected from *C. carpio* brooders of three different age groups ranged between 2-2.5 ml and the density of milt varied considerably between the three age groups. Significant variations ($p < 0.05$) were noticed among the dilution ratios also. From Fig. 1, it can be seen that the mean highest density of $1.107 \pm 0.13 \times 10^{10}$ cells ml⁻¹ was recorded in 6 month age group brooders when diluted at a ratio of 1:40.

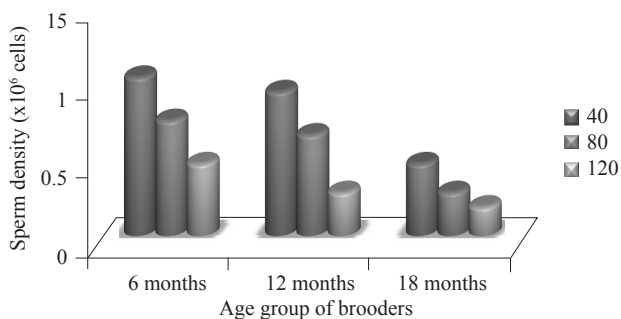


Fig. 1. Relation between age group, dilution ratios and density of *C. carpio* spermatozoa

Highest (<0.05) spermatocrit value was observed for milt collected from 6 months old brooder (84±2.5%) while the lowest value of 73±1.5% was found in 18 months old brooder (Fig. 2). The Ca²⁺ and Mg²⁺ levels in the seminal plasma of common carp significantly (p<0.05) increased as age increased. (Table 1).

Fecundity varied significantly between brooders of the three age groups as depicted in Fig. 3. The lowest fecundity (71 eggs per g body weight of fish) was observed in 6 months old brooder, whereas, 18 months old brooder had highest fecundity of 162 eggs per g body weight.

The mean motility duration of fresh milt collected from 6, 12 and 18 months old brooders were 65.89±1.52, 73±2 and 62.33±1.02 sec respectively with motility score of 10 and 100% live cells (Table 1). However, from Table 2 it is evident that the initial mean motility duration

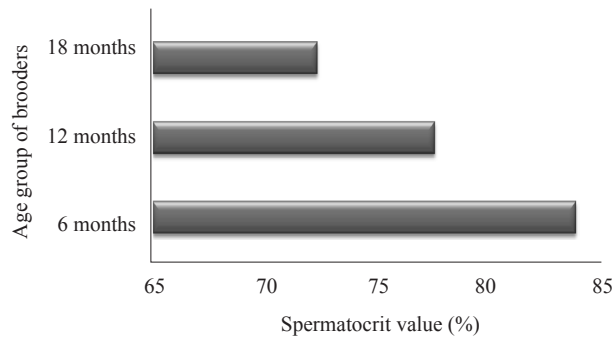


Fig. 2. Spermatocrit value (%) of milt collected from three age group brooders of *C. carpio*

Table 1. Spermatological parameters of fresh milt collected from common carp brooders of three age groups

Parameters	Age group		
	6 months	12 months	18 months
Motility duration (sec)	65.89±1.52	73±2	62.33±1.02
Motility score	10	10	10
Live cells (%)	100	100	100
Dead cells (%)	0	0	0
Spermatocrit value (%)	84	78	73
Calcium (mg l ⁻¹)	57	64	69
Magnesium (mg l ⁻¹)	51	58	63

Table 2. Motility duration (s) and score of milt diluted with freshwater fish saline

Dilution ratio	Age group					
	6 months		12 months		18 months	
	Duration	Score	Duration	Score	Duration	Score
1:40	61.66±1.52	9	69.66±4.72*	10	59±4	9
1:80	60.33±1.15	9	66.66±3.78	10	56.33±3.05	9
1:120	57±1	9	53±5	10	54.66±2.51	9

(p<0.05)

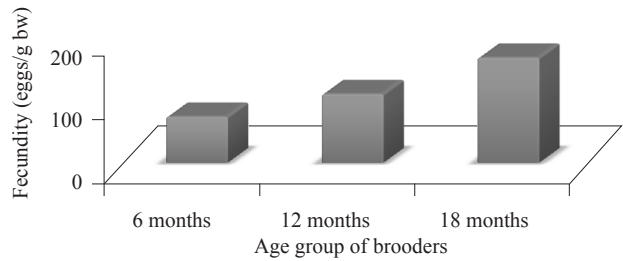


Fig. 3. Effect of age on the fecundity of *C. carpio* brooders

of the spermatozoa of fresh milt was affected when the milt was diluted at different dilution ratios.

The mean motility duration values recorded were significantly different (p<0.05) between milt collected from brooders of different age groups with highest duration (69.66±4.72 sec) observed in the milt collected from 12 months old brooder. As it can be seen from (Table 1), the duration was the lowest in 18 months old brooder. Among the dilution ratios, 1:40 alone gave the highest mean motility duration in all the age groups. The motility score was 10 in milt produced by 12 months old brooder whereas it was 9 in other two age group brooders (Table 2).

Highest mean fertilisation percentage of 91.3±3.05% was obtained from 12 months old brooders (dilution ratio 1:40) with mean hatching rate of 76.5±2.64%. Brooders of 18 months old exhibited lowest mean fertilisation rate of 67.8±4.5% at 1:120 dilution ratio. The lowest mean hatching rate was produced by 6 months old brooder with milt diluted at 1:120 ratio (Table 3). The fertilisation and hatching rates were significantly (p<0.05) different between different age group brooders and dilution ratios.

Discussion

Sperm density of common carp collected from brooders of three age groups and diluted at three dilution ratios, ranged between 0.2 and 1.1×10¹⁰ cells ml⁻¹ of milt. Six months old brooders had milt with high density of 1.107±0.13×10¹⁰ cells ml⁻¹. However, the lowest sperm density (0.5±1.43×10¹⁰ cells ml⁻¹) was produced by 18 months old brooder (Fig. 1). From these observations, it is clear that sperm density decreased with increase in

Table 3. Fertilisation rate (FR) and hatching rate (HR) of eggs fertilised with milt from common carp brooders of three age groups at three dilution ratios

Dilution ratio	Age group					
	6 months		12 months		18 months	
	FR (%)	HR (%)	FR (%)	HR (%)	FR (%)	HR (%)
1:40	79.2±2.51	54.1±1.15	91.3±3.05*	76.5±2.64*	72.6±2	68.8±3.51
1:80	75.5±1.52	61.3±3	89.1±3	73.9±1.73	68.6±3.78	62.3±2.08
1:120	69.1±2.08	49.8±1.52	86.6±1.52	68.2±1	67.8±4.5	57.4±1.52

(p<0.05)

age as opined by Tekin *et al.* (2003) and Aliniya *et al.* (2013). Variations in the sperm density might be due to differences in individuals, fish size and season as noted by Glogowski *et al.* (1999). Spermatozoa concentration is known to vary with respect to different age groups (Zuromska, 1981; Buyukhatipoglu and Holtz, 1984).

The highest spermatocrit value of 84±2.5% was found in 6 months old brooder. The value was lower in 18 months old brooder (73±1.5%) as it could be seen from Fig. 2. The results fall in line with Liley *et al.* (2002) and Tekin *et al.* (2003) who reported that spermatocrit value decreases with increasing age.

The Mg²⁺ value in the present study was in the range of 51-63 mg l⁻¹ and Ca²⁺ value was in the range of 57-69 mg l⁻¹ (Table 1). The values were significantly different between different age group of brooders (p<0.05). The results are similar to the reports by Cierieszko *et al.* (2000) who found that calcium and magnesium ions ranged from 1 to 2 mM. In the present study, the Mg²⁺ level in the milt was 63 mg l⁻¹ in 18 months old brooder which decreased to 51 mg l⁻¹ in 6 months old brooder. This is in agreement with the findings of Aas *et al.* (1991) that composition of seminal fluid changes in respect to age. Ca²⁺ level in the study were at its maximum of 69 mg l⁻¹ in 18 months old brooder whereas it was only 57 mg l⁻¹ in 6 months old brooder. It is in accordance with Chitsaz *et al.* (2012) who mentioned that as age increases, calcium level in the seminal plasma of common carp also increases and the increase was significant. Biochemical evaluation of seminal plasma is an important criterion for assessment of milt quality (Billard *et al.*, 1995). Ca²⁺ and Mg²⁺ are important components in the seminal plasma of common carp. Freezing and thawing causes an increase of Na⁺ and Ca²⁺ and decrease of K⁺ and Mg²⁺ which might be a possible reason for reduction in the fertilising capacity of cryopreserved spermatozoa (Kurokura *et al.*, 1980).

The fecundity of common carp in the three age groups varied significantly (p<0.05). Fecundity of 6 months old brooder was 71 eggs per g body weight of fish whereas it was 106 eggs per g body weight at

12 months and 162 eggs per g body weight at 18 months (Fig. 3). This increase in fecundity is as reported by Reznick *et al.* (2002) and are similar to that reported by Parameswaran *et al.* (1972) who observed the fecundity of farmed common carp in India as around 15500 eggs per kg body weight. Similarly, Dobriyal *et al.* (1990) reported the fecundity of common carp to be in the range of 9927-104884 eggs per kg body weight.

The mean motility duration of fresh milt collected from 6, 12 and 18 months old brooders were 65.89±1.52, 73±2 and 62.33±1.02 sec respectively (Table 1). These differences in motility duration of undiluted milt samples was supported by Keeran and Woods (2002) who stated that the mean percentages of motile sperm obtained from the initially collected fresh, undiluted milt changed significantly over the course of the spawning season.

The highest mean motility duration of fresh milt of 73±2 sec was noticed in milt collected from 12 months old brooder. Upon dilution with FFS at a ratio of 1:40, the value dropped to the mean motility duration of 72.3±3.0 sec. This can be vouched by the fact that motility decreases immediately after dilution (Sahin *et al.*, 2013; Mostafapor and Ardebili, 2014).

Motility duration was highest in milt collected from 12 months old brooder (Table 2). The reason for this might be the age of the brooders as mentioned by Vuthiphandchai and Zohar (1999). Reduction in milt quality throughout the spawning season is a common factor (Legendre and Billard, 1980; Piironen, 1985; Munkittrick and Moccia, 1987; Aas *et al.*, 1991) due to aging of spermatozoa (Rana, 1995; Suquet *et al.*, 1998; Babiak, 2006). Legendre and Billard (1980) reported that the ability of spermatozoa to tolerate the stress of freezing and thawing may be altered during the course of spawning.

In the present study, the Ca²⁺ and Mg²⁺ values were higher for 18 months old brooder. From the reports it can be seen that, motility duration increases when there is increase in Ca²⁺ level (Alavi and Cosson, 2006; Islam and Akhtar, 2011; Khara *et al.*, 2014). Contradictory to this, the motility duration was higher in 12 months old brooder (73±2 sec) for which the Ca²⁺ level was 64 mg l⁻¹.

Brooders of 18 months had Ca^{2+} level of 69 mg l^{-1} with motility duration of $62.33 \pm 1.02 \text{ sec}$ (Table 1). No seasonal influence was observed in the present study because common carp may spawn throughout the year in tropical areas of India, with peaks in January-March and July-August (FAO). The milt collection from 6, 12 and 18 months old brooder falls in the months of March, September and April respectively. Hence the spermatological properties of milt from brooders of different age group are not influenced by season.

From Table 2, it could be said that milt diluted at 1:40 ratio alone exhibited high motility duration. This is because at higher cell concentrations, the motility of milt significantly decreases, which is attributed to cell compression because of limited intracellular space (Sader *et al.*, 2011). In support to this, Jing *et al.* (2009) reported that motility decreased with increased dilution.

Highest mean fertilisation ($91.3 \pm 3.05\%$) and mean hatching rates ($76.5 \pm 2.64\%$) were obtained from 12 months old brooders, when milt was diluted at 1:40 ratio. Other age group brooders and dilution ratios produced comparatively low fertilisation/hatching rates and the values were significantly different ($p < 0.05$). The difference in the fertilisation and hatching percentages between brooders of different age groups might be due to the poor quality of milt from 6 and 18 months old brooders which directly reflected on low fertilisation and hatching rates as discussed by various researchers (Methven and Crim, 1991; Shangguan and Crim, 1995; Suquet *et al.*, 1998).

The fertilisation rate was found to decrease with increasing dilution ratios. This fact was vouched by Cognie *et al.* (1989) who found that the motility and fertility of deep frozen spermatozoa of *C. carpio* were significantly improved when the dilution ratio was reduced from 1:100 to 1:2. According to Rana and Mc Andrew (1989) and Gwo *et al.* (1991) the milt dilution ratio had strong effect on fertility. There was significant difference ($p < 0.05$) between the fertilisation and hatching rates of different age group brooders and between different dilution ratios.

Hence based on the present study it is advised to use common carp males and females of 12 months age for higher fertilisation success as they produced good quality milt in terms of motility duration, fertilisation and hatching rates.

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