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Assessment of Sperm Concentration and Determination of Optimum Sperm to Egg Ratio in *Heteropneustes fossilis* (Bloch)

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Abstract

Determination of optimum sperm requirements in artificial breeding helps to improve the fertilization efficiency, avoids wastage of sperm and thus minimizes the sacrificing of males for milt. Quantification of sperm cells was done using a spectrophotometer at 420 nm followed by haemocytometer counting. Best correlation (r^2 = 0.97) was recorded. The minimum number of sperm cells required for optimal fertilization success in *Heteropneustes fossilis* was determined. Fertilization success of 78 to 93% was recorded at $8x10^3$ to $8x10^7$ sperm per egg. The highest fertilization success of 98.18% was recorded at $8x10^7$ spermatozoa per egg.

Introduction

The stinging catfish (*Heteropneustes fossilis*) is a commercially important freshwater catfish distributed in the tropical waters of the Indian

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sub-continent. In *H. fossilis* there are considerable studies on the reproductive biology, breeding and cryopreservation of sperm, but there are only very few studies on gamete management. Specifically, there is no study on optimum requirement of sperm to egg ratio. The optimum sperm requirements for fertilization success was studied in rainbow trout and brown trout (Billard 1982), pike (Erdal and Graham 1987), guppy (Billard and Cosson 1992), halibut (quoted in Suquet et al. 1995), European catfish (Linhart et al. 1997) sturgeon (Persov 1953), Atlantic croaker (Gwo et al. 1991), turbot (Suquet et al. 1995) and seabass (Fauvel et al. 1999). The standardization of the sperm to egg ratio is a prerequisite for improving sperm diluents and thereby minimizing the wastage of sperm, especially where the male brooders have to be sacrificed for their sperm. Thus, the objective of this study is to standardize a technique for a quick assessment of sperm number, spectrometrically and to determine the optimum sperm to egg ratio, to improve fertilization efficiency and reduce wastage of sperm in artificial breeding.

Materials and Methods

Brood stock collection

The experimental fish *H. fossilis* (males, 35 – 50 g body weight (BW) and females, 80 – 120 g BW) were collected during the month of June (early spawning phase) from the ponds in and around Alwarkurichi (8°48'00"N; 77°27'30"E). Upon arrival at the laboratory, fish were disinfected with formalin (50 ppm) for 2 hours to remove external parasites and pathogens. Mature female brood fish were primarily selected on the basis of soft and bulging belly, which yielded continuous ova of uniform size on gentle stripping. Then these eggs were examined under a stereozoom microscope for checking of their maturity. Brooders with fully mature preovulatory follicles (spherical, translucent, greenish brown ova) were selected. These selected fish were stocked into the polyvinylchloride (PVC) tank (3x2x1 m) with continuous aeration, normal photoperiod (13.5 h L : 10.5 h D) and an ambient water temperature (26 ±1°C) for inducing final ovulation.

Induction of ovulation

The induction of final maturation and ovulation was stimulated, using a single intramuscular injection of Ovaprim (marketed by Glaxo Smith

Kline Pharmaceuticals Limited, each ml contains salmon gonadotropin releasing hormone 20mcg and Domperidone 10 mcg), at the rate of 0.3 and 0.5 mg. kg^{-1} body weight for males and females, respectively.

Collection of Gametes

The eggs were collected after 10 hours of hormonal injection at an ambient water temperature of 26±1°C (determined by pilot studies), by gently pressing the abdomen towards the anus which yields a copious stream of transparent greenish brown eggs which were collected in a glass bowl previously rinsed with Hanks Balanced Salt Solution (HBSS) [Himedia].

The males were sacrificed at random for the collection of milt. The testes were surgically removed and were cut into small bits, then gently squeezed in a glass tissue homogenizer (1 g / 1 ml) with HBSS. The remaining tissue particles were removed and the milt was diluted to 1:10 with HBSS. Sperm motility was checked for each sample by taking an aliquot of diluted sperm of 10 µl in a micro slide and activated by 3 µl of tap water, then viewed under a microscope at X400. Samples with high motility alone were used for the experiment.

Assessment of sperm concentration

Sperm numbers was assessed using spectrophotometry (Spectronic Genesys, USA) and haemocytometer (Neubaur Improved) (Suquet et al. 1992). The sperm suspension was diluted with HBSS in the ratio of 1:500, which brought spermatozoa to counting concentration. The absorption spectrum of the diluted sperm was measured at 420 nm followed by haemocytometer counting by two observers. The correlation between absorbance and sperm concentration was analyzed. There was no significant difference between observers count. These values were analyzed for correlation coefficient.

Determination of sperm to egg ratio

Constant volume of fresh sperm suspension was prepared following the desired concentration ranging from 2.4×10^3 to 2.4×10^{10} per 0.5 ml.. The sperm suspension was deposited over 300±40 eggs. The sperm suspension was gently mixed for uniform distribution. Then 0.2 ml of tap water was added for sperm activation. Activated spermatozoa were allowed to fertilize the eggs for 5 minutes. Then the excess sperm were washed out repeatedly with fresh water and the fertilized eggs were transferred to plastic trays for incubation. The resulting fertilization rate was

assessed under the stereozoom microscope (Nikon SZU, Japan) for 50 randomly chosen eggs. Eggs were assumed to be fertilized when they exhibit the 8-cell stage with regular division after 45 minutes (unfertilized egg may divide but with irregular divisions). The success in the form of fertilization percentage was recorded. This experiment was repeated four times in each sperm concentration.

Results

Spectrophotometric Evaluation

Quantification of spermatozoa using haemocytometer needs a counting concentration, which was brought by diluting the sperm suspension with HBSS for different ratios (1:100, 1:200, 1:300, 1:400, 1:500 and 1:600). The best countable dilution for assessing sperm concentration was 1:500. A linear relationship with a highly significant correlation was found between sperm numbers assessed using haemocytometer counting and its optical density using simple linear regression (Fig. 1). Best correlation was recorded ($r^2 = 0.97$).

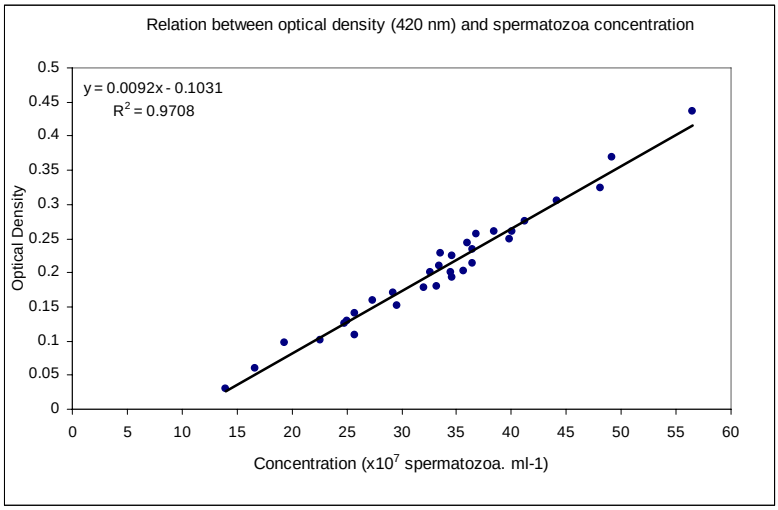

Figure 1. Diluted sperm (1:500 HBSS) number assessed by haemocytometer counting and its optical density using linear regression

Sperm to egg ratio

The number of sperm per eggs was plotted against percentage of fertilization in figure 2. It was observed that the increase in sperm availability from 8 to 8 x 10^7 sperms per egg resulted in a rapid increase in fertilization rate. It was observed that the fertilization rate was low (8, 21 and 39%) in 8, 80 and 800 spermatozoa•egg^{-1}, respectively. Then there was a progressive increase (78 to 93%) in fertilization rate from 8,000 to 80,000,000 spermatozoa•egg^{-1}. The highest fertilization success of 98.18% was also recorded at 8 x 10^7 spermatozoa•egg^{-1}. There was no significant difference (P>0.1) between 8,000 to 80,000,000 spermatozoa•egg^{-1}. From these results it is clear that 8,000 spermatozoa•egg^{-1} are required for optimum fertilization success.

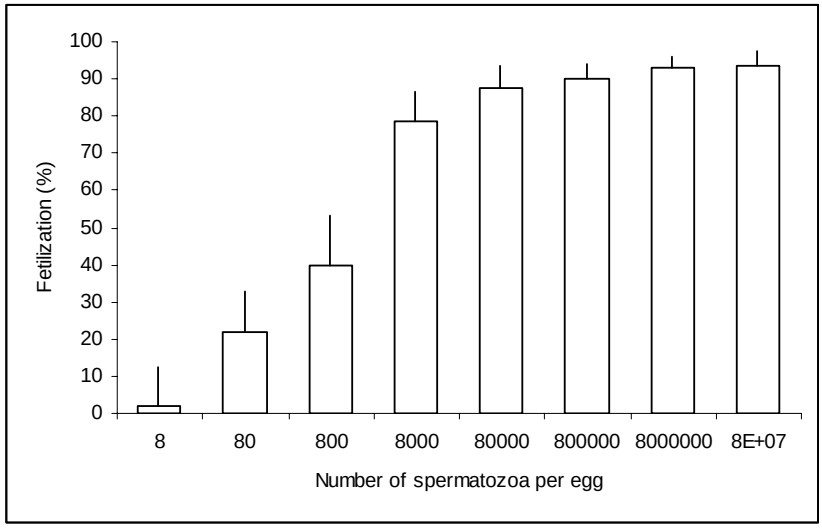

Figure 2. Mean fertilization percentage with increasing number of sperm to egg ratio. Mean values of four replicates are shown, vertical lines are standard deviations

Discussion

This is the first study involving *H. fossilis* to assess sperm concentration and determine optimal sperm requirements for egg fertilization. Milt concentration has been assessed by three techniques: counting in a haemocytometer chamber (Buyukhatipoglu and Holtz 1984; Leung-Trujillo and Lawrence 1987), spermatocrit (Munkittrick and Moccia 1987) and spectrophotometric evaluation (Suquet et al. 1992). Among the three

techniques, spectrophotometry is the simplest and fastest. Spectrophotometry was more reliably used for the assessment of sperm concentration in different species, viz., rainbow trout and common carp (Billard et al. 1971), turbot (Suquet et al. 1992), seabass (Fauvel et al. 1999), white and yellow perch (Ciereszko and Dabrowski 1993). The observation maximum (420 nm) was chosen for the best correlation between absorbance and sperm concentration evaluated by haemocytometer with minimal interference (Suquet et al.1992). The milt was diluted several times due to much higher spermatozoa concentration (Ciereszko and Dabrowski 1993). In the present study, for countable sperm number the milt was diluted at 1:100 to 1:600 and the best countable dilution was found to be at 1:500. The linear relationship between optical density and sperm concentration offers a rapid method for estimation of spermatozoa number in *H. fossilis* with significant correlation co-efficient $r^2 = 0.97$. Similar correlation was reported in rainbow trout (Billard et al. 1971), turbot (Suquet et al. 1992) and seabass (Fauvel et al. 1999).

Optimum fertilization rate was observed from 8,000 to 80,000,000 spermatozoa•egg-^{1}. There is no significant difference ($P>0.1$) from 8×10^3 to 8×10^7 spermatozoa•egg-^{1}. This sperm requirement is low when compared to the results reported in species, viz, European catfish, where the required spermatozoa•egg-^{1} was estimated at 40,000 (Redonodo et al. 1989); in sturgeon, 43,000 (Persov 1953 as cited by Suquet et al. 1995), in brown trout, 43,000 (Erdal and Graham 1987) and in sea bass, 66,000 (Fauvel et al. 1999). At the same time, this minimum requirement of 8,000 sperm per egg is higher when compared to a few other species such as 1,000 in Atlantic croaker (Gwo et al. 1991), and 6,000 in turbot (Suquet et al. 1995). The minimum sperm (8,000) requirement for high fertilization success in *H. fossilis* indicates high fertilizing ability of *H. fossilis* sperm. Other specific feature of *H. fossilis* gametes were the smaller egg size ranging from 0.95 to 1.2 mm, when compared to the egg size of carp at 1.4 mm (Linhart et al, 1995), pike at 2 mm and rainbow trout at 4 mm (Billard 1982), 3 mm in halibut (Haug 1990). This consequently attains the probability of sperm reaching the micropyle to be higher by traveling a shorter distance compared to other fish species. Further, an attracting phenomenon which guides spermatozoa to the micropyle may also be present in *H. fossilis*.

From this study it is concluded that the sperm cell concentration can be easily assessed spectrophotometrically at 420 nm in 1:500 dilution with HBSS. For optimum fertilization success, a minimum of 8,000 sper-

matozoa•egg-^{1} is sufficient. Thus the sperm cells can be utilized to the maximum.

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