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Optimization of artificial fertilization in the stinging catfish *Heteropneustes fossilis* (Bloch)

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Summary

Determination of optimum sperm requirement in artificial breeding helps to improve the fertilization efficiency, sperm economy and minimize the sacrificing of males for milt. Quantification of sperm cells was done by spectrophotometer at 420 nm followed by haemocytometer counting. Best correlation ($r^2 = 0.97$) was recorded. The minimum number of sperm required for optimal fertilization success in *Heteropneustes fossilis* was determined. An average fertilization success of 78–93% was recorded at 8×10^3 to 8×10^7 sperm per egg. The optimum contact duration of gametes was 5 minutes. The highest fertilization rate of 98.18% was recorded at 8×10^7 spermatozoa.egg^{-1}.

Keywords: Artificial breeding, Fertilization, Optimal sperm requirement, Sperm assessment, Sperm-to-egg ratio

Introduction

The stinging catfish (*Heteropneustes fossilis*) is a commercially important freshwater catfish distributed in the tropical waters of the Indian sub-continent. In *H. fossilis* there is considerable studies on the reproductive biology (Manjula & Malancha, 1999; Saxena & Sandhu, 2005), breeding (Tripathi, 1996) and cryopreservation of sperm (Lal *et al.*, 2009), but there are only a very few studies on gamete management. Especially there is no study on optimum requirement of sperm-to-egg ratio. The optimum sperm requirement for fertilization success was studied in rainbow trout and brown trout (Billard, 1982), pike (Erdal & Graham, 1987), guppy (Billard & 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 sea-bass (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 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). On arrival to the laboratory, fish were disinfected with formalin (50 ppm) for 2 h to remove external parasites and pathogens. Matured 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 its maturity. Ovaries that contained fully mature preovulatory follicles are spherical, translucent, green

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to brown in colour with centrally located germinal vesicle and lacking previtelline space, such brooders alone were selected. These selected fish were stocked to the polyvinylchloride (PVC) tank ($3 \times 2 \times 1$ m) with continuous aeration, normal photoperiod (13.5 h light (L):10.5 h dark (D)) and an ambient water temperature ($26 \pm 1^{\circ}$C) for inducing final ovulation.

Induction of ovulation

The induction of final maturation and ovulation was stimulated, using a single intramuscular injection of ovaprim, at the rate of 0.3 and 0.5 mg.kg^{-1} body weight for males and females.

Collection of gametes

The eggs were collected after 10 h of hormonal injection at an ambient water temperature of $26 \pm 1^{\circ}$C (determined by pilot studies), by gently pressing the abdomen towards the anus, which yields a copious stream of transparent greenish brown eggs and were collected in a glass bowl previously rinsed with Hanks Balanced Salt Solution (HBSS) [Hi-media].

The males were anesthetized and sacrificed for the collection of milt. The testes were surgically removed and were cut into small bits, then gently squeezed in a glass tissue homogenizer with a few drops of HBSS. The left out tissue particles were removed and the milt was diluted to 1:10 with HBSS. The sperm motility was checked for each sample, by taking an aliquot of 10 µl in a microslide and activated by 3 µl of tap water, then viewed under microscope at ×400. Samples with high motility alone were used for the experiment.

Assessment of sperm concentration

Sperm numbers were assessed by spectrophotometry (Spectronic Genesys) and haemocytometer (Marks) (Suquet *et al.*, 1992). The sperm suspension was diluted with HBSS in the ration 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 analysed. There was no significant difference between observers' counts. These values were analyzed for correlation coefficient.

Determination of sperm-to-egg ratio and gametes contact time

Constant volume of fresh sperm suspension was prepared with desired concentration ranging from 2.4×10^2 to 2.4×10^7 per 0.5 ml was prepared. The sperm suspension was deposited over 300 ± 25 eggs. The sperm suspension was gently mixed for uniform distribution. Then 0.2 ml of tap water was added for sperm activation. Activated sperms were allowed to fertilize the eggs for three different durations such as 3, 5 and 7 minutes, all in separate batches. 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) for a 50 randomly chosen eggs. Eggs were assumed to be fertilized when they exhibited the 8-cell stage (after 45 min). The successes in the form of fertilization percentage were recorded. This experiment was repeated four times in each sperm concentration.

Results

Spectrophotometric evaluation

Quantification of spermatozoa using a 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 highly significant correlation was found between sperm number assessed by haemocytometer counting and its optical density using simple linear regression (Fig. 1). Best correlation was recorded ($r^2 = 0.97$).

Sperm-to-egg ratio and contact time

The number of sperm per egg is plotted against percentage of fertilization in Fig. 2. It is observed that the increase in sperm availability from 8 to 8×10^7 sperm per egg resulted in a progressive increase in fertilization rate. It is observed that the fertilization rate was low (8, 21 and 39%) in 8, 80 and 800 spermatozoa/egg respectively. Then there was a progressive increase (78 to 93%) in fertilization rate from 8000 to 80,000,000 spermatozoa.egg^{-1}. The highest fertilization success of 98.18% was also recorded at 8×10^7 spermatozoa.egg^{-1}. There is no significant difference ($p > 0.1$) between 8000 to 80,000,000 spermatozoa.egg^{-1}. From this result it is clear that 8000 spermatozoa.egg^{-1} is required for optimum fertilization success. As for the gametes contact time, 5 minutes was found to be the most optimum duration (Fig. 2). When the contact time was reduced to 3 minutes, fertilization percentage decreased.

Discussion

This is the first study in *H. fossilis* to assess the sperm concentration and determine optimal sperm requirement for egg fertilization. Milt concentration has been assessed by three techniques, counting in a

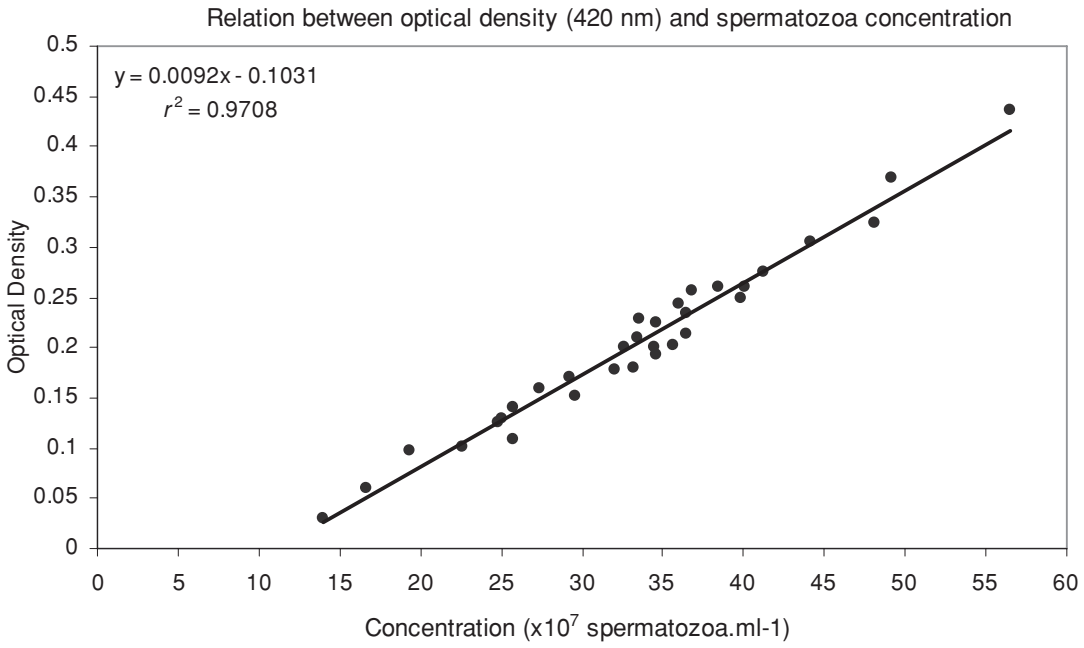

Figure 1 Sperm number assessed by haemocytometer counting and its optical density using linear regression.

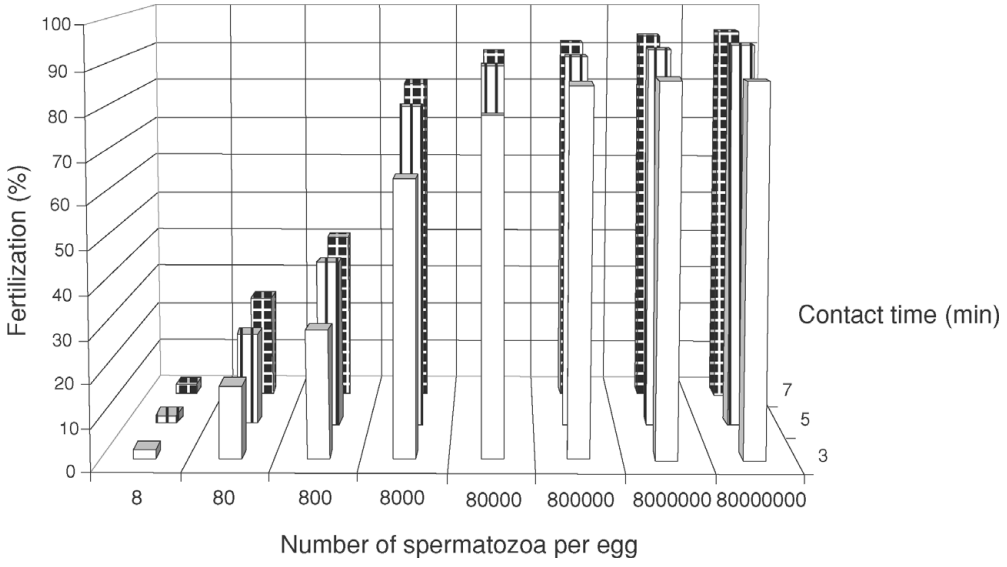

Figure 2 Mean fertilization percentage with increasing number of sperm-to-egg ratio and their contact time.

haemocytometer chamber (Buyukhatipoglu & Holtz, 1984; Leung-Trujillo & Lawrence, 1987), spermatocrit (Munkittrick & Moccia, 1987) and spectrophotometric evaluation (Suquet *et al*., 1992). Among the three techniques, spectrophotometry is simple and faster. 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 & 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 is being diluted several times due to much higher spermatozoa concentration (Ciereszko & 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 8000 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 with the results reported in species, viz, European catfish, where the required spermatozoa.egg^{-1} was estimated as 40,000 (Redono *et al*., 1989); in sturgeon, 43,000 (Persov, 1953; coated in Suquet *et al*., 1995), in brown trout, 43,000 (Edral & Graham, 1987) and in sea bass, 66,000 (Fauvel *et al*., 1999). At the same time, this minimum requirement of 8000 sperm per egg is higher when compared with few other species such as 1000 in Atlantic croaker (Gwo *et al*., 1991) as 6000 in turbot (Suquet *et al*., 1995). The minimum sperm (8000) 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 0.95 to 1.2 mm, when compared with the egg size of rainbow trout, 3 mm (Billard and Cosson, 1992); carp, 4.8 mm (Perchec, 1992; coated in Suquet *et al*., 1995); pike, 2 mm and trout, 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*. The gametes contact time of 5 minutes duration for fertilization is likely the function of an increase probability of successful encounter between gametes. Fertilization success as a function of time has been observed in turbot (Suquet *et al*. 1995). However extending the time above 5 minutes didn't increase fertilization percentage, showing the life span of *H. fossilis* sperm.

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 an optimum fertilization success a minimum of 8000 spermatozoa.egg^{-1} is sufficient, with a contact duration of five minutes. Thus the sperm cells can be utilized to the maximum.

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