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Note

Microplate-based incubation method for quality assessment of eggs of Indian major carp *Catla catla*

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Abstract

Incubation of fertilised eggs from the Indian major carp species, *Catla catla* was carried out in tissue culture microplates to assess the quality in terms of hatching rate and post-hatch survival rate. Eggs from five females (F1, F2, F3, F5 and F6) were strip spawned and fertilised with milt from a single male. The fertilised eggs were distributed at one egg per well of 24 well microplates, with three plates for each female. The plates were incubated at 30°C in an incubator with continuous shaking and complete hatching was observed by 36 h. The hatching rate varied within a range of 0 to 52.8% showing the maximum of 5% for F5 followed by F1 (50%) which were significantly higher than the other three females (F2-18.1, F6-5.6 and F3-0%). The eggs from these two females (F1 and F5) thus could be considered as good. The survival percentage of hatchlings recorded for a period of 8 days post-hatching was found to vary from 58.3 to 83.3 and there was low variability among different females on any observed day. Between these two egg quality determinants, the hatching rate was found to be more suitable considering the wide variability shown by different females indicating the quality difference. The microplate method seems to be an appropriate tool to assess the egg quality in catla and can be extended to other species as well.

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Indian major carps (IMCs) constitute the bulk of freshwater aquaculture production in India. Among IMCs, *Catla catla* is one of the most preferred species due to its large size, higher growth rate and taste. Mostly the species is cultured in South and South-east Asian countries (FAO, 2022). Although the seed of this species is now produced in large quantities in hatcheries, poor quality is regarded as the major constraint to the success of its culture. To get quality seeds, the production of large numbers of high quality spawns is a primary prerequisite. Compared to sperm/milt, the quality of spawns is primarily determined by maternal effects with good quality ovulated eggs (Trippel *et al.,* 2005). Egg quality can be defined as the ability of the egg to be fertilised and subsequently develop into a normal embryo (Bobe, 2015). As developing embryos and freshly-hatched larvae are quite sensitive during this phase, proper environmental conditions must be provided for incubation and hatching, simulating the natural conditions (Korwin-Kossakowski, 2012).

Poor egg quality leads to a number of problems during early developmental stages such as lack of fertilisation, problems of egg activation, developmental arrest and embryonic deformities (Bobe, 2015). Hence, assessment of egg quality is very much crucial to prevent poor quality spawns from entering to the culture system. Egg quality is usually evaluated by several determinants such as fertilisation rate, hatching rate and spawn survival rate besides many morphological, biophysical and biochemical parameters (Bromage *et al.,* 1994). The most reliable indicator of good egg quality, however, is high larval survival, through egg hatching and the yolk sac stage of larval development before exogenous feeding is required (Duncan *et al.,* 2013). In the conventional method, these parameters are estimated by stocking tens to hundreds of fertilised eggs in a glass beaker or petridish and counting dead larvae every 12-24 h (Dasgupta *et al.,* 2009; Unuma *et al.,* 2019). An alternative method to egg quality assessment is the microplate method wherein individual

eggs are put into each well of 24/48/96 well plates and incubated at a suitable temperature for fertilisation followed by hatching and survival till first feeding. This method was originally developed by Shields *et al.* (1997) with eggs from Atlantic halibut, *Hippoglossus hippoglossus* and has later been successfully applied in many fish species such as the gilthead seabream (*Sparus aurata*), white seabream (*Diplodus sargus*) and European seabass (*Dicentrarchus labrax*) (Panini *et al.,* 2001), Japanese eel (*Anguilla japonic*a) (Unuma *et al.,* 2004), fathead minnow (*Pimephales promelas*) (Marentette *et al.,* 2015) and barfin flounder (*Verasper moseri*) (Unuma *et al.,* 2019). Besides egg quality, microplate incubation has also been employed for toxicity testing on fish embryos (Perovic *et al.,* 2013; Melo *et al.,* 2015).

Normally, the egg quality parameters are estimated after eggs are fertilised with milt and the developmental success of larva is monitored. However, a prior biomarker type indicator would facilitate early identification of quality and hence would be possible to avoid unnecessary spawn production resulting in mortality. Advanced methods like transcriptomics and proteomics have been tried to find such predictive biomarkers in eggs (Migaud *et al.,* 2013; Reading *et al.,* 2018). A similar proteomic method is planned for the detection of biomarkers of egg quality in catla. However, having a set of good or poor quality eggs for proteomic comparison has to be based on the earlier mentioned parameters. Our experience with catla eggs indicates the lack of reproducibility of the conventional method in quality assessment. Besides, the fertilisation rate in catla usually has been seen to be more than 80% without much variability between females. Hence, to achieve an accurate estimate of egg quality parameters particularly hatching rate or hatchling survival percentage, a microplate method was developed in the present study.

Breeding of *C. catla* was carried out in a circular hatchery in the ICAR-Central Institute of Freshwater Aquaculture (CIFA) campus for family generation for selection under catla selective breeding programme. Both matured male and female brooders maintained in one pond in the CIFA farm were collected and left separately in circular tanks (size: 3 m dia) for conditioning. They were induced-bred with the synthetic hormone, ovatide (dose: 0.5 ml kg^{-1} body weight of female and 0.35 ml kg^{-1} body weight of male) injected intraperitoneally at the base of the pectoral fin and left separately for ovulation. After around 6-7 h, the females and males were stripped individually in dry plastic trays and plastic sample bottles to collect eggs and milt, respectively. Thereafter, 10 ml of eggs from one female was fertilised *in vitro* with 1 ml of milt from a single male. Both were thoroughly mixed in plastic trays with the help of a bird feather and the mixing continued for approximately 10 min further after addition of water for fertilisation. The eggs were gradually washed with pond water till individual eggs were separate and were transferred to the laboratory for microplate experiments.

The collected eggs were washed with filtered (Whatman no. 1 filter paper) and well-aerated (dissolved oxygen 5 ppm) pond water two times. pH of the medium was within 7.6-7.8 and hardness within 65-70 ppm. The fertilised eggs were visually differentiated from unfertilised eggs by their transparency with a clearly visible nucleus at the centre. Fertilised eggs were carefully collected with the help of a spatula and slowly released into the wells (one egg per well) of tissue culture plates (HiMedia, India), filled with pond water (2 ml per well). The plates were then incubated at 30°C and observed daily for hatching and larval survival. Initially, the following incubation conditions were standardised: 1. Microplate with 24 wells (2 ml water per well) *vs* 48 wells (1 ml water per well) (HiMedia, India); 2. Autoclaved pond water *vs*. pond water filtered with Whatman no. 1 filter paper; 3. Incubation of microplate in BOD incubator (M.K. Scientific Instruments, India) without shaking vs incubation in shaking incubator (Daihan LabTech Co. Ltd., Korea); 4. Incubation of microplate with cover *vs* without cover and 5. Microplate hatching with water exchange on alternate days *vs* without water exchange.

Based on the results of the standardisation experiments, the final experiment was conducted in 24 well microplates with egg incubation in filtered pond water (2 ml per well) in a shaking incubator (100 rpm) at 30°C and with regular water exchanges done on alternate days. Here, eggs collected from 5 females (10 ml each) belonging to different selectively-bred families (F1, F2, F3, F5 and F6) were fertilised *in vitro* with 1 ml milt each from a single male (M2). The fertilised eggs were distributed individually in microplate wells. The plates were kept open in plastic trays and incubated as mentioned above. The plates were observed daily for 10 days and hatching, as well as the survival of hatchlings, were recorded for assessing the egg quality. As one of our objectives was to look for larval survival as a reflection of egg quality through maternal effects, the larvae were not provided with external food during the extended experimental period (beyond 3 days post-hatch) to avoid influence on survival rate. For each female sample, three plates were used as replicates. The hatching rate and the hatchling survival rate on a particular day after hatching were calculated as per the following formulae:

Hatching rate: No. of hatched larvae/Total number of eggs incubated x 100

Survival rate: Total no. of hatchlings survived/Total number of live hatchlings x 100

The eggs incubated in each microplate was considered as a replicate and the resulting data pertaining to hatching rate and survival percentage were calculated. Data were presented as mean±SE. Data on hatching percentage were further subjected to one way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at a significance level of $p<0.05$.

The fertilised eggs of all the catla females in microplates were observed daily for analysing their hatching and survival rate up to ten days (Fig. 1). Hatching of larvae was completed by 36 h post-incubation and the hatching rates of all the five females were calculated. Maximum hatching percentage was found in female F5 (52.8), followed by F1 (50.0) which were significantly higher than F2 (18.1), F6 (5.6) and F3 (0.0) (Table 1). The hatchlings were found normal in appearance and quite active. No abnormal or deformed hatchlings was detected in these microplate experiments. The survival percentage of hatched-out larvae in four females were recorded for a period of 8 days post-hatching and found to vary from 58.3 to 83.3 (Table 2). However, the highest survival percentage was seen with F6, where there was the lowest hatching rate. Besides, there were less variations in survival rate among females on any particular day observed during the study. Further, it was observed that mostly the larval mortality occurred within the initial 2/3 days post-hatching and subsequently, the survival was more stable. Again after 6 days post-hatching, mortality occurred possibly due to lack of proper nutrition.

Assessing egg quality in cultivable fishes is very much crucial to select good quality spawn to enter the culture system from an economic standpoint. Thus, in this study, we used multiwell tissue culture plates

(a) (b)

Fig. 1. Hatching of fertilised catla eggs in microplates: a. Microplate showing distribution of fertilised eggs and b. Microplate showing hatchlings

Table 1. Hatching percentage of eggs from different catla females during microplate incubation

Female number	Eggs incubated	Eggs hatched 36 hpf	Hatching %	*Mean hatching %
F1	24	9	37.5	
	24	15	62.5	50.0 ± 7.2[a]
	24	12	50.0	
F2	24	6	25.0	
	24	4	16.7	18.1 ± 3.7[b]
	24	3	12.5	
F3	24	0	0.0	
	24	0	0.0	0.0 ± 0.0[c]
	24	0	0.0	
F5	24	13	54.2	
	24	11	45.8	52.8 ± 3.7[a]
	24	14	58.3	
F6	24	2	8.3	
	24	1	4.2	5.6 ± 1.4[bc]
	24	1	4.2	

*Data represents mean±SE. Means with different superscripts are significantly different (ANOVA, DMRT, p<0.05).

Table 2. Survival percentage of hatchlings from different catla females during microplate incubation for a period of 8 days post-hatch. Data represent mean±SE of 3 microplate results (total hatchlings survived/total numbers of hatchlings in three microplates)

Days	F1	F2	F5	F6
0 dph	100.0±0.0 (36/36)	100.0±0.0 (13/13)	100.0±0.0 (38/38)	100.0±0.0 (4/4)
1 dph	91.9±4.1 (33/36)	100.0±0.0 (13/13)	92.9±7.1 (35/38)	100.0±0.0 (4/4)
2 dph	91.9±4.1 (33/36)	88.9 ±11.1 (12/13)	90.3±6.3 (34/38)	100.0±0.0 (4/4)
3 dph	82.2±9.7 (30/36)	80.6±10.0 (11/13)	79.1±1.4 (30/38)	100.0±0.0 (4/4)
4 dph	79.5±7.2 (29/36)	80.6±10.0 (11/13)	79.1±1.4 (30/38)	100.0±0.0 (4/4)
5 dph	73.5±10.4 (27/36)	80.6±10.0 (11/13)	79.1±1.4 (30/38)	100.0±0.0 (4/4)
6 dph	73.5±10.4 (27/36)	80.6±10.0 (11/13)	79.1±1.4 (30/38)	100.0±0.0 (4/4)
7 dph	68.5±8.0 (25/36)	63.9±15.5 (9/13)	79.1±1.4 (30/38)	83.3±16.7 (3/4)
8 dph	63.0 ±3.7 (23/36)	58.3±12.7 (8/13)	76.5±3.8 (30/38)	83.3±16.7 (3/4)

for analysing the quality of catla eggs. The major advantages of this method includes: Individual incubation of eggs avoiding the negative effects such as a decline in water quality caused by dead eggs (Nakata *et al.,* 2004) and the ease of monitoring the same eggs and larvae over a period of time (Panini *et al.,* 2001). Use of microplates for egg quality assessment has successfully been conducted in a variety of marine (Shields *et al.,* 1997; Panini *et al.,* 2001; Unuma *et al.,* 2004; Unuma *et al.,* 2019) and a few freshwater fish species such as fathead minnow *Pimephales promelas* (Marentette *et al.*, 2015) and silver perch *Bidyanus bidyanus* (Sulaeman and Fotedar, 2017). Since the quality of developing embryo and spawn is primarily dependent on maternal factors (Trippel *et al.,* 2005), we tried to establish the quality of eggs from different females. Milt from a single male was used in our experiment to nullify the sperm effect on embryo/spawn quality. The fertilised eggs were incubated in a shaking incubator with continuous shaking since the agitation of catla eggs for hatching is a required event in the normal incubation process. Marentette *et al.* (2015) have similarly employed an orbital shaker for continuous agitation that showed significantly increased growth of fathead minnow, *P. promelas* embryos compared to non-agitated multi-well plates. The temperature of incubation was

selected as 30^{0}C and complete hatching was observed by 36 h of incubation. Rahman *et al.* (2020) have earlier used a temperature range of 28-30^{o}C for fertilised catla eggs, which showed maximum hatching by 34-40 h. The most cumbersome aspect of this experiment is putting individual eggs into microplates. However, the advantages of getting a proper estimation of egg quality outweigh the disadvantages of putting individual eggs in microplates as similarly opined by Panini *et al.* (2001).

The hatching rate of eggs is considered as one of the ultimate measures of egg quality in fish species (Brooks *et al.,* 1997). In the present study, the hatching rate was found to vary widely between 0 to 52.8% indicating the different qualities of eggs. Highly variable hatching ranging from 0 to 80.4% has been observed with Japanese eel, *Anguilla japonica* eggs in microplates (Unuma *et al.,* 2004). However, high hatchability of 95 to 100% has been observed with fathead minnow, *P. promelas* eggs in microplates (Marentette *et al.,* 2015). These differences could possibly be due to species-specific differences and hence, the hatching percentage must be evaluated with each concerned species as an egg quality marker. The larvae hatched in these small volume wells were normal in appearance and agility. Survival from hatching for a period of 8 days however, was less variable among different females to be graded as good or poor. The mortality in hatchlings was typically observed in the initial and later days of incubation. The mortality in the initial phase could be attributed to the weak hatchlings developed from the eggs. In catla, the yolk sac remains up to 3 d post-hatch (Sheriff and Altaff, 2018) and hence, the later phase mortality possibly indicates lack of nutrients after yolk absorption. A similar observation has been made by Unuma *et al.* (2019) that the barfin flounder *V. moseri* larvae survived more than 2 weeks in microplates after complete yolk resorption until they were starved to death. From the entire study, it is concluded that assessing the eggs for hatching percentage showing a broad range of variation is possibly a better indicator of egg quality than the hatchling survival which has a narrow range of variation. Another advantage with hatching percentage is that, here it is possible to incubate the eggs without water exchange since time period required to obtain the results is only 36 h. The application of this standardised microplate technique could be extended for egg quality assessment in other freshwater fish species, with prior validation. Besides egg quality assessment, this microplate methodology may also be a valuable tool to assess the effects of toxicological compounds on individual hatchlings in a regulated manner.

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