Generation of viable progeny from dead brooders of endangered catfish *Clarias magur* (Hamilton, 1822)

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**Abstract**

The obligatory air-breathing catfish *Clarias magur* is a prime candidate for aquaculture owing to its unique taste, high growth rate, and hardy nature. However, recently the IUCN has listed the species under the endangered category because the population has critically declined in the wild. The sexually mature *C. magur* brooders are often collected from their natural habitats for seed production in captivity. In many cases, the brooder dies due to handling injuries or confinement stress. In this study, we demonstrated that viable progeny could be generated from freshly dead sexually mature *C. magur*. Three hours after death, the gonads were excised, macroscopically examined and gamete viability was evaluated. Artificial fertilization was performed by mixing the sperm suspension with the eggs. Water was added after 1 min of mixing to activate the fertilization process. We observed 85%-93% fertilization success from gametes derived from dead donors as opposed to 90%-95% from those derived from live control donors. The embryos showed normal development and resulted in the generation of 88%-92% viable progeny, which was similar to the progeny derived from control donors (92%-93%). The results obtained in this study will have profound implications in enhancing the seed production of endangered *C. magur* and could potentially be applied to other key commercially or endangered fish species.
Keywords: Biological sciences, Developmental biology, Zoology

1. Introduction

The endangered air-breathing catfish *Clarias magur* is widely distributed in the Ganga and Brahmaputra river basins in northern and north-eastern India, Nepal, Bhutan, and Bangladesh (Ng and Kottelat, 2008). However, according to recent IUCN assessment, the species has become endangered due to various known and unknown factors (The IUCN Red List, 2017). In the coming years, the species might further decline critically, if appropriate management strategies are not implemented to revive their natural population (Vishwanath, 2010).

Currently, mass propagation of *C. magur* through aquaculture is limited because of very low availability of seed for stocking. Often, for aquaculture the seeds are collected from wild sources such as wetlands, ponds, swamps (Sahoo et al., 2006). However, such collection does not guarantee the purity of species because they produce viable hybrids through mating with other species, such as *C. gariepinus* and *C. macrocephalus*, in the wild (Na-Nakorn et al., 2004; Das, 2002). The major bottleneck in mass scale seed production in captivity is the poor response to synthetic hormones for gonadal maturation and spawning (Sahoo et al., 2008). Alternatively, artificial fertilization is usually practised to produce the hatchlings by sacrificing male for harvesting spermatozoa cell. One disadvantage of this method is that the male *C. magur* can contribute the genetic material only once in its lifetime. This results in reduced effective population size. Currently, we are working to develop nonsacrificial methods, through biotechnological interventions, for the smooth release of spermatozoa.

*C. magur* is a bottom dweller and prefers shallow water bodies (Hossain et al., 2006). During the breeding season, the species is usually caught from natural habitat by netting and using indigenously built traps and transported to hatchery units often in numbers exceeding the recommended stocking density (Mahapatra et al., 2000). This invariably induces body injuries, causing the death in many cases. Such dead animals, irrespective of maturity status, are often discarded and only the healthy live individuals are used for breeding purposes. In this study, we have tested the hypothesis that gametes obtained from sexually mature *C. magur* dead for up to 3 h can be effectively used for generation of a viable progeny. The results suggest that using gametes from dead donors can be a viable proposition to be applied in some circumstances to other commercial or endangered fish species.
2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Ethics Committee of ICAR-National Bureau of Fish Genetic Resources. All the fishes used in the experiments were handled according to the prescribed guidelines.

2.2. Animals and rearing protocol

The sexually mature adult *C. magur* (mean body weight ± SD of 220 ± 9.5 g for males and 250 ± 11 g for females) used in the experiment were procured locally in July and reared in 500 L fiber reinforced plastic tank at a density of 7.5 kg of fish per m$^3$ at the rearing facilities of the ICAR-National Bureau of Fish Genetic Resources, Lucknow, India. The animals were reared at 25 ± 2 °C under a constant light cycle (12L:12D) and fed with pelleted commercial diet (ABIS Export India Private Limited) two times per day to satiation.

2.3. Artificial fertilization trial

Eight fish were found dead 2 days after collection from natural habitats. The dead animals, time of death (3 h) confirmed from the data retrieved from surveillance camera, were immediately recovered from the tank and within 30 min gametes were used for artificial fertilization trials. Three pairs of donor males and females were selected on the basis of their morphological condition for maturity (Sinha et al., 2014). Briefly, males were selected on the basis of long pointed genital papilla (Fig. 1A'; arrowhead), whereas females were selected on the basis of round and reddish genital papilla with bulging abdomen (Fig. 1A; arrow). The body weight was recorded, and the gonads were excised, macroscopically examined, photographed using a digital camera, and weighed to the nearest 0.01 g for measuring gonadosomatic index (GSI). A sperm suspension from the dead donor males was prepared by grinding the testes in physiological saline solution (0.9% NaCl solution; pH 7.0) in 1:4 ratio. The ovaries were dissected carefully, and the eggs were transferred to a dry plastic tray. One millilitre of sperm suspension prepared from a donor male, containing approximately $2.5 \times 10^4$ cells, was poured on 250–300 numbers of eggs derived from a donor female and gently mixed with the help of soft bird feather. A 50 ml freshwater (Temperature: 25 ± 2 °C; pH: 7.5; Hardness: 45 ppm) was added after 1 min of mixing to activate the fertilization process. Ten minutes post activation, the translucent eggs containing embryonic eyes were considered fertilized. Unfertilized eggs were removed immediately from the tray to prevent fouling of water. For live control, males (n = 2) and females (n = 2) were artificially fertilized separately and embryos were incubated in flow through system similar to the condition where embryos from dead donors were incubated.
Fig. 1. Ventral view of *Clarias magur* female (A) and male (A’) recovered 3 h post death. Note that the female had round and reddish genital papilla (arrow), whereas the male had long pointed genital papilla (arrowhead). The animals had died due to severe injuries during transportation (highlighted). The sexual
2.4. Viability assessment

The viability of the spermatozoa cells was determined using the Trypan blue exclusion test (Irfan et al., 1998). Trypan blue stains dead cells blue but does not permeate the membranes of living cells, which, therefore, remain unstained (Majhi et al., 2014). Accordingly, 0.5 mL of 0.4% Trypan blue solution (Himedia, India) was added to 0.5 mL of cell suspension in 2 mL tubes and mixed thoroughly. The mixture was allowed to stand for 10 min at room temperature and then centrifuged at 500 g at 4 °C for 10 min. Supernatants were discarded and the pellets resuspended in 1 mL freshwater Ringer’s solution (Fluck, 1995) containing 0.5% bovine serum albumin (Sigma Aldrich, USA). A small droplet from the tube was transferred to a cell counter and observed under a microscope (Leica DMi8) at 60X. The percentage of viable cells was calculated counting the number of dead and live cells in each field. Cells that stained blue were considered dead. This procedure was repeated three times for each sample.

2.5. Hatching protocol and embryonic development

The fertilized embryos were transferred to a water flow-through system, indigenously built using plastic tubs (diameter: 12 cm; height: 6 cm) and a glass aquarium. Briefly, water from an overhead glass tank passed through the hatching tubs placed at the center on a glass platform by gravity and drained into a collection tank placed at the bottom. The flow of water was adjusted by regulator fitted in each tub. The fertilized eggs are uniformly distributed in the plastic tubs and a feeble current of water (2.5 liter per hour) was provided to maintain good water quality. Fertilized eggs were incubated at 30 ± 1 °C in flow through system and hatchlings were counted after 24 h. Monitoring was performed at regular intervals for embryonic development using a stereo zoom microscope (Leica S8 APO) and dead embryos, if found, discarded manually. The observations on embryonic development recorded in this study (water temperature: 30 ± 1 °C) was compared with previous studies (25–29 °C) to evaluate embryonic development based on water temperature.

2.6. Statistical analysis

The measured parameters such as gonadosomatic index (GSI), fertilization and hatching percentage were compared among the individuals through one-way analysis of variance (ANOVA) with the Mann–Whitney test by using Graphpad prism ver.7.00 for windows (Graphpad software, San Diego, Carlifornia, USA). Maturity of the dead donors was further assessed by the morphological evaluation of ovary (B) and testis (B’). Scale bar-3 cm (A,A’), 2 cm (B,B’).
The differences between the groups were considered statistically significant at $P < 0.05$.

3. Results and discussion

In this study we found that gametes from sexually mature *Clarias magur* dead for three hours, could be used for the generation of viable progeny through artificial fertilization.

In teleost fishes, the GSI is consider as a proxy to measure the gonadal maturation status (Brewer et al., 2008). In this investigation, the GSI value we recorded for
dead females and males were 10.5 ± 0.5% and 0.29 ± 0.01%, respectively (Fig. 1B-B’ and Fig. 2). These value of GSI from dead donors was similar to that of sexually mature live control female (11 ± 0.3%) and male (0.3 ± 0.01%), which were ready for breeding (Thakur, 1978). The GSI in teleost fish varies during the three distinct phase of reproduction such as early maturation, maturation and post maturation (Thakur, 1978). For example, in case of C. magur, the GSI of males varies in the range 0.2%–0.25% during breeding season and in the ranges 0.08%–0.1% and 0.03%–0.05% during early maturation and post maturation phase, respectively (Thakur, 1978; Mahapatra et al., 2000). Similarly, in females, fully matured individuals have a GSI in the range 10.5%–11% and during early maturation and post maturation the GSI ranges are 0.5%–0.6% and 0.3%–0.4%, respectively (Hossain et al., 2006; Thakur, 1978). The GSI value we obtained in this study for the dead donors of C. magur were in the range of the maturation phase. Moreover, when we subjected the gametes for viability assessment, 85% of male gametes were found to be viable even after 3 h of death as oppose to 90% in control male. The artificial fertilization between gametes derived from dead donors resulted in 85–93% fertilization success (Table 1). This rate was similar to the fertilization rate obtained from live controls i.e. 90%–95%.

The fertilized embryos derived from dead C. magur donors and live controls were incubated at 30 ± 1 °C in a flow through system to observe the embryonic development (Fig. 3C-I). The stages of embryonic development are summarized in Table 2. Our observation suggested no difference in the stages of embryonic development between the two groups. However, the only difference observed was early attainment of developmental stages in our case, compared with previous reports (Thakur, 1980). For example, at 30 ± 1 °C water temperature we observed the 2-cells stage at 35 min after the fertilization (Fig. 3D). Similarly, the somite stage were observed after 15 h (Fig. 3E), and the twitching movement of the embryos started 19 h after fertilization (Fig. 3F). More frequent twitching movement and in few embryos with free tail ends (Fig. 3G) were observed at 20 h

**Table 1.** Results of artificial fertilization of eggs and sperm derived from dead *Clarias magur* donors (#1 to #3; recovered three hours after death) and live control (#4 and #5). Note that the embryos derived from both the group were reared at 30 ± 1 °C water temperature. Column with different superscript varies significantly (Mann-Whitney test, *P* < 0.05).

| Male *C. magur* donor | Eggs (n) from female *C. magur* donor | Fertilization (n; %) | Hatching (n; %) |
|-----------------------|--------------------------------------|---------------------|-----------------|
| #1                    | 240                                  | 215 (89)*           | 195 (90)*       |
| #2                    | 450                                  | 420 (93)*           | 390 (92)*       |
| #3                    | 380                                  | 323 (85)*           | 285 (88)*       |
| Control (#4)          | 410                                  | 370 (90)*           | 345 (93)*       |
| Control (#5)          | 350                                  | 335 (95)*           | 310 (92)*       |

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30 min after the fertilization. The larvae started hatching out after 21 h of fertilization, and all the larvae hatched out within 30 min from the beginning of hatching process (Fig. 3H). However, the same stages of *C. magur* embryos incubated at water temperature of 25–29 °C was observed to be delayed by 15 min (2-cells stage) to 1 h (somite stage) after the fertilization (Table 2). Usually, such differences in embryonic development in teleost fish species occur because of the

![Developmental stages of embryo derived from dead donors of *Clarias magur*. Note that the embryos were reared at 30 ± 1 °C water temperature and compared with the result of normal *C. magur* embryonic development obtained at water temperature of 25–29 °C (Thakur, 1980).](image)

### Table 2. Developmental stages of embryos derived from dead donors of *Clarias magur*. Note, the embryos were reared at 30 ± 1 °C water temperature and compared with the result of normal *C. magur* embryonic development obtained at water temperature of 25–29 °C (Thakur, 1980).

| Developmental stages                                      | Time after fertilization (in this case) | Time after fertilization (Thakur, 1980) |
|-----------------------------------------------------------|----------------------------------------|----------------------------------------|
| Fertilized eggs                                           | 0 h 0 min                              | 0 h 0 min                              |
| 2-cells                                                   | 0 h 35 min                             | 0 h 45 min                             |
| 4-cells                                                   | 0 h 50 min                             | 1 h 5 min                              |
| 8-cells                                                   | 1 h 0 min                              | 1 h 20 min                             |
| Many cell                                                 | 2 h 10 min                             | 2 h 30 min                             |
| Morula                                                    | 3 h 0 min                              | 3 h 30 min                             |
| Germinal ring formation                                   | 4 h 0 min                              | 5 h 0 min                              |
| Embryo formation                                          | 6 h 10 min                             | 7 h 30 min                             |
| Differentiation of head and tail ends of embryo           | 09 h 0 min                             | 10 h 0 min                             |
| Somite differentiation                                     | 11 h 0 min                             | 12 h 0 min                             |
| 8-somite                                                  | 12 h 30 min                            | 14 h 30 min                            |
| 12-somite                                                 | 15 h 0 min                             | 16 h 0 min                             |
| Kupfer's vesicle formation                                | 17 h 0 min                             | 18 h 0 min                             |
| 25-somite                                                 | 17 h 40 min                            | 19 h 0 min                             |
| Kuper's vesicle disappear                                 | 18 h 30 min                            | 20 h 0 min                             |
| Twitching movement start                                  | 19 h 30 min                            | 20 h 30 min                            |
| 40-somite                                                 | 20 h 0 min                             | 21 h 30 min                            |
| Twitching movement more vigorous                          | 20 h 30 min                            | 21 h 45 min                            |
| Egg membrane rupture and hatching of larvae               | 21 h 0 min                             | 21 h 55 min                            |

Fig. 3. Developmental stages of embryo derived from dead donors of *Clarias magur*. Note that the embryos were reared at 30 ± 1 °C water temperature for hatching. A) Spread of spermatozoa derived from dead male donor. B) Spread of eggs derived from dead female donor C) view of a fertilized embryo soon after the activation of fertilization process. D) 2-cells stage embryo recovered at 35 min after fertilization. E) at 12 h 30 min after the fertilization, the embryo attained the somite stage. F) the twitching movement began at 19 h 30 min after the fertilization G) more vigorous twitching movement was observed at 20 h 30 min H) the membrane began to rupture at 21 h after fertilization and all the larvae hatched out within 30 min from the beginning of the hatching process. I) at 15 days after hatching, the progeny derived from the dead donors exhibited active vertical and horizontal movement similar to progeny derived from live control. Scale bar—50 μm (A,B), 500 μm (C-H), 10 cm (I).
incubation water temperature (Olaniyi and Omitogun, 2014); the higher the water temperature, the faster is the embryonic development and hatching (Hogendoorn and Vismans, 1980). The most salient result obtained in this study is successful hatching of 88%–92% embryos obtained from dead donors compared to 92%–93% from live control. Moreover, 15 days after hatching, the progeny derived from the dead parents were observed to have active vertical and horizontal movement in the nursery tank (Fig. 3I), which is a behavioral characteristic exhibited by healthy normal progeny. This observation is critical for increasing the seed production and aquaculture of endangered C. magur because they are discarded, if found dead, without using the genetic materials. Here we suggested that such male and female broods of C. magur could be used for seed production even after their death. These results are consistent with Koteeswaran and Pandian (2002), who reported that male Heteropneustes fossilis (teleostean) could be post-mortem preserved for 240 days under refrigerated condition (−20 °C) and successfully used the sperm for fertilizing freshly derived eggs, resulting in healthy progeny. However, the only striking difference between our report and previous reports is that we obtained viable gametes from parents recovered three hours post death and stored at non-refrigerated condition. The information we report here is crucial, particularly when the broods are transported over long distances to the seed production unit and, on the way, the animals succumb to injury or stress. Nevertheless, it remains to be investigated how long the gametes from such dead brooders retain their functional viability at normal and freezing temperature.

In conclusion, the proposition we made in this study, that is, using dead brooders of C. magur for the generation of viable progeny will certainly contribute to increasing seed production of this endangered species. It is worth mentioning that, the seed production of this catfish for aquaculture is largely based on wild collected brood stock because of the lack of complete domestication of this species. The findings of this study indicate that the post mortality brood fish could be effectively used for seed production, rather than discarding them. We believe that such attempts should also be made in other valuable fish species that incur injuries during collection and die after a few hours. This will not only ensure appropriate utilization of genetic materials for generation of progeny but will also prevent economic loss of hatchery units that invest resources in raising the brood stock for seed production.

**Declarations**

**Author contribution statement**

Sullip Kumar Majhi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Santosh Kumar: Performed the experiments; Analyzed and interpreted the data.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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