**Polyandrous Fertilization Enhances Offspring Survival Rate In An Indian Major Carp *Labeo Rohita*.**

**Running Title:** Polyandry increases larval survival

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**Abstract**

Fish, like most other animals, follow different mating patterns (e.g. polyandry, monandry, etc.) to have direct (non-genetic) or indirect (genetic) benefits and therefore, this study was carried out to explore whether the monandrous or polyandrous fertilization strategy could provide more reproductive benefits in the hatchery production of an important aquaculture species, the Indian major carp (*Labeo rohita*). The study found no significant differences in hatching rate, survival rate and deformation rate of hatchlings, standard length and body area of offspring between polyandrous and monoandrous groups. The findings, however, revealed that polyandrous fertilization ensured significantly higher offspring survival rate than monandrous group. This study confirms that fish breeders and other associated stakeholders can obtain more benefits by following the polyandrous fertilization strategy, which can ensure good quality larvae for successful aquaculture.

**Keywords:** Polyandry; Monandry; Fish reproduction; Non-genetic benefit; Offspring fitness

**Introduction**

Polyandrous fertilization is practiced in many fish hatcheries around the world where pooled milt from multiple males is mixed with a single female’s eggs (Kekäläinen *et al.* 2010; Lumley *et al.* 2016). This fertilization strategy is usually followed to obtain non-genetic (Lewis and Pitcher 2017; Squires *et al.* 2012) and genetic benefits ( Kekäläinen *et al.* 2010; Sagebakken *et al*. 2011). In many species of different taxa, polyandrous females produce eggs with higher in number, smaller in size, greater in viability and larger in yolk volume (Kawazu *et al.* 2017; Omkar and Pandey 2010; Ward 2000) that ensure higher fertilization and hatching success (Byrne and Whiting 2008; Jennions *et al.* 2007). Evidence also shows that polyandrous females produce offspring having comparatively larger body size (Maklakov and Lubin 2006) and higher survival rate (Croshaw *et al.* 2017) than the monandrous one.

The underlying mechanisms of these benefits are thought to be mediated through good genes (Yasui 1997), sperm competition (Firman and Simmons 2008) and sperm-egg interaction (Evans and Sherman 2013) Non-genetic benefits are comparatively easy to quantify, while genetic benefits demonstration faces a lot of challenges that need to consider all the possible factors influencing offspring fitness. Although many studies in different taxa already unveiled that polyandry can enhance offspring fitness, only a limited number of studies conducted to explore the influence of polyandry on fish offspring fitness (Kekäläinen *et al.* 2010; Sagebakken *et al.* 2011) and to date, no result has been found on this issue in a commercially important aquaculture species. Therefore, this study was carried out to explore whether polyandrous fertilization strategy could provide any benefit to the fish breeders of a commercially important Indian major carp (*Labeo rohita*, Hamilton 1822).

The Indian major carp (*L. rohita*) is one of the most important aquaculture species in the Indian sub-continents which was produced 1,843 tonnes (3% of world aquaculture finfish production) in 2016 (FAO 2018). Millions of people are engaged throughout its production system where a large number of hatcheries are in operation to produce larvae for the culture of this species. The poor quality of eggs and milt, lower rate of fertilization and hatching, poor larval quality, etc. are the major problems facing these hatcheries (Mohan 2007; Sahoo *et al.* 2017). The polyandrous fertilization technique could be an alternative option together with other strategies (e.g. broodstock management, genetic selection, etc.) to mitigate these loses.

**Materials and Methods**

**Experimental approaches**

Sexually mature same sized 30 males (mean±SE: 1.59±0.05 kg) and 10 females (mean±SE: 1.33±0.02 kg) were sorted out for this study to conduct a full-sib and half-sib breeding experiment (Figure 1). The recommended doses of pituitary glands were applied for induced spawning (Jhingran and Pullin 1985). The experiment was conducted during the first natural spawning season to have good quality of gametes (Chattopadhyay 2017), while collection and mixing of milt and eggs in all trials were done at the same time to avoid sequential effects (Khara 2015).

After the fertilization, the total number of alive hatchlings per incubator was counted and stocked them according to family for three days to wean gradually exogenous feeding. Then the total number of alive and visually deformed hatchlings was counted and recorded from which 30 good offspring were reared family-wise in a glass aquarium (50cm×29cm×30cm) for two weeks to assess their fitness. The dissolved oxygen (DO) and pH of water were checked daily. The offspring were fed to their apparent satiation level twice daily (Rahman *et al.* 2020). Finally, the offspring number was counted to estimate the survival rate. Then a photograph of each anaesthetized offspring was taken using the digital camera (Canon DS126621) to measure total length and body area by using the *ImageJ* software v-1.46 (<https://imagej.nih.gov/ij/download.html>). The study was carried out up to this larval stage because local farmers practise this system for nursing, larval rearing, and marketing purposes (Rahman *et al.* 2020).

**Statistical analysis**

The ‘R’ software (version 3.6.3) was used for the statistical analysis (R Development Core Team 2020). The Shapiro-Wilk test of normality and Levene's tests for homogeneity of variance were applied using the ‘onewaytests’ package. For any comparison of a measured trait between two fertilization groups, the analysis of variance (ANOVA) was applied (using the ‘car’ package) for normally distributed and homogenous traits, whereas the Kruskal-Wallis (K-W) method was followed for traits of not normally distributed by any transformation but homogenous, and the Welch test was performed (using the ‘car’ package) for a non-normalized and non-homogenized data.

The linear and nonlinear mixed effects or NLME model was applied using the ‘nlme’ package to have the ‘maximum likelihood (ML)’ for comparison(Pinheiro *et al.* 2019). In the model, fertilization group was included as a ‘fixed factor’, males and females body weight and their interaction (males:females body weight) were fixed as ‘covariates’, while the males (sire) and females (dam) IDs were incorporated as ‘random effects’. The ML test showed the *p*-values of the random effects by comparing the models. To avoid pitfalls of significance testing, the Cohen’s effect size was calculated (Cohen 1988) using ‘MuMIn’ package. Finally, all other graphs were made using the ‘ggplot2’ package.

**Results**

The analysis found no significant differences in males’ body weight (ANOVA: F1,38=0.001, *p*=0.99), standard length (ANOVA: F1,38=0.007, *p*=0.93) and milt weight (ANOVA: F1,38=0.1, *p*=0.92) used between two fertilization groups. Similarly common females showed no significant variations in body weight (K-W: χ2=0, *p*=1.0), standard length (K-W: χ2=0, *p*=1.0), egg weight (K-W: χ2=0, *p*=1.0), egg number (ANOVA: F1,38=1.34, *p*=0.25) and egg diameter (K-W: χ2=0, *p*=1.0).

The NLME model revealed no significant variations in hatching and their deformation rate (Table 1). Interestingly, a significant difference (t1,35=2.08, *p*<0.05) was found in offspring survival rate between these two groups (Figure 2), while no significant variations were observed in offspring total length and body area (Table 1). The marginal effect size (R2m=0.16) of the model clearly showed the mean difference distribution between two fertilization groups with a bootstrap of 95% confidence interval (Figure 3) which is sample size independent displaying all observed values and avoiding false dichotomy.

**Discussion**

In the present study, broodstocks’ size, feed quality and amount, and induced spawning protocols were maintained strictly same to minimize any variation because of these factors. Each fish was handled very carefully to avoid any physiological stress. Moreover, the breeding protocols and random selection of the same sized parents tried to minimize their effects. However, parental genetic quality, egg-sperm interaction and parental non-genetic materials might be the plausible reasons for the higher offspring survival in polyandrous group.

In ‘good genes hypothesis’, males vary in their genetic quality which is the main interest of females to mate with (Cutrera *et al.* 2012). Unfortunately, females are unable to assess these genes directly (Neff 2000) and therefore, they prefer to mate with multiple males to achieve the highest benefits from the superior males (Jennions and Petrie 2000). Evidence shows that superior males produce good quality sperm which have higher paternity success through sperm competition (Gage *et al.* 2004) as well as increase the offspring fitness (Eilertsen *et al.* 2009). In the present study, the higher offspring survival in polyandrous group could be because of sperm competition in which superior males might fertilize the maximum number of eggs. Unfortunately, the present study failed to assess the sperm traits because of the very remote location of hatchery which has very limited laboratory facilities. Moreover, sperm concentration was not possible to count because of high fat contents. At this point, total milt quantity was taken as a good indicator of male’s quality considering the suggestions of other studies (Kowalski and Cejko 2019; Rahman *et al.* 2020).

Evidence has shown that polyandrous strategy can ensure inbreeding avoidance (Michalczyk *et al.* 2011) and increase outbreeding (Burdfield-Steel *et al.* 2015) which are usually the outcomes of sperm-by- eggs interactions (Alonzo *et al.* 2016; Evans *et al.* 2011). Studies have revealed that ovarian fluid and gamete-recognition proteins can modulate fertilization success of genetically compatible males (Evans and Sherman 2013). Thus, egg-sperm interaction during fertilization could be responsible for higher offspring survival in polyandrous group.

Parents can transfer non-genetic information (e.g. chromatin modifications, RNAs and proteins) to offspring through gametes (Casas and Vavouri 2014; Giesing *et al.* 2011) which play important roles in offspring fitness and development. In European whitefish, offspring of low temperature treated milt became larger and swimmed better than those of high temperature group (Kekäläinen *et al.* 2018). In three-spined sticklebacks, offspring of predator-exposed mothers exhibited tighter shoaling behavior than those of no predator-exposed mothers (Giesing *et al.* 2011). Thus, it could be possible in the present study that parental non-genetic information might influence the offspring survival. However, further studies are needed to explore how (underlying mechanisms) and why (genetic or non-genetic purposes) do they prefer polyandrous rather than monandrous reproductive tactics?

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**Author Contributions**

Md. Moshiur Rahman, Muhammad Abdur Rouf and Sk. Mustafizur Rahman designed the experiment. Soma Kundu and Md. Shahin Parvez conducted the experiment and collected the data. Md. Moshiur Rahman, Md. Shahin Parvez and Muhammad Abdur Rouf performed the analysis. Md. Moshiur Rahman, Md. Asaduzzaman, Md. Mostafizur Rahman, Roshmon Thomas Mathew, Yousef Ahmed Alkhamis and Sheikh. Mustafizur Rahman prepared the draft manuscript, and also provided extensive support and feedback on further data analysis and finalized the manuscript. All authors commented manuscript drafts.

**Conflicts of Interest**

Authors declare no conflicts of interest.

**Data Availability**

We hereby declare that data related to this study are available with the corresponding author and will be produced on demand.

**Ethics Approval**

This work was carried out under the School of Life Science of Khulna University’s Animal Ethics approval (KUAEC-2019/07/8).

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**Figures**

**Fig. 1:** The overall experimental design shows total number of broodstocks (i.e. 30 males and 10 females), and their spawning and larval rearing processes after diving them into two fertilization groups (e.g. monandry and polyandry). The entire spawning process was divided into 10 batches in which milt from three males and eggs from single female were used during each batch. Total 10 trials were conducted to obtain the data from 40 families. After collecting in clean and dry bowls, weights of total milt and eggs were measured and stirred well to have random samples. 2 mL eggs was collected from each female with a new syringe, and immediately 1.5 mL milt was collected with a new syringe and mixed with the eggs for monandrous fertilization, while 0.5 mL milt from each male was collected for polyandrous group (‘R’ indicates - replication). 1 mL eggs were collected in tubes for counting and imaging later. The fertilized egg masses were transferred to previously labeled plastic containers (2 L) fitted with continuous aerated water flow and kept for incubation at ambient temperature until the maximum hatching occurred. 30 larvae from each replication per family were reared up to two weeks to assess their fitness (‘n’ indicates total number of larvae per fertilization group).



**Fig. 2:** The offspring survival rate (%) between two fertilization groups where ‘M’ (M1-M30) on the top of each bar denotes the respective male ID and ‘F’ (F1-F10) indicates the common female ID, while the number at the bottom of each bar is the family ID (1-40).



**Fig. 3:** The estimation plot of offspring survival rate model displaying the marginal effect size with a mean difference between two fertilization group of *Labeo rohita.*

**Table**

**Table 1:** Outcomes of the linear and nonlinear mixed effects (NLME) models showing the differences in reproductive performance between two fertilization groups of *Labeo rohita* during this study. In the model*,* DF*-* degrees of freedom, S.E- standard error, S.D- standard deviation and L-ratio- likelihood ratio. Significant values are denoted as Italic and bold at the level of *p* < 0.05

|  |  |
| --- | --- |
| Response trait | Estimates of variables |
|  |
| Hatching rate (%) | *Fixed effect* | *Estimates* | *S.E* | *DF* | *t-value* | *p* |
| Fertilization group | 0.13 | 0.16 | 35 | 0.81 | 0.42 |
| Males body weight (kg) | -4.74 | 2.38 | 35 | -1.99 | 0.05 |
| Females body weight (kg) | -3.20 | 2.06 | 35 | -1.56 | 0.13 |
| Males:females body weight | 2.49 | 1.32 | 35 | 1.89 | 0.07 |
| *Random effect* | *Variance* | *S.D* |  - | *L-ratio* | *p* |
| Males ID | 0.08 | 0.28 |  | 0.00 | 1 |
| Females ID | 0.08 | 0.28 |  | 0.00 | 1 |
| Residuals | 0.01 | 0.11 |  |  |  |
|  |
| Hatchling deformation rate (%) | *Fixed effect* | *Estimates* | *S.E* | *DF* | *t-value* | *p* |
| Fertilization group | -0.24 | 0.21 | 35 | -1.14 | 0.26 |
| Males body weight (kg) | -0.14 | 3.10 | 35 | -0.04 | 0.97 |
| Females body weight (kg) | -1.23 | 2.68 | 35 | -0.46 | 0.65 |
| Males:females body weight | 0.25 | 1.72 | 35 | -0.15 | 0.88 |
| *Random effect* | *Variance* | *S.D* |  - | *L-ratio* | *p* |
| Males ID | 0.14 | 0.37 |  | 0.00 | 1 |
| Females ID | 0.14 | 0.37 |  | 0.00 | 1 |
| Residuals | 0.02 | 0.14 |  |  |  |
|  |
| Offspring survival rate (%) | *Fixed effect* | *Estimates* | *S.E* | *DF* | *t-value* | *p* |
| Fertilization group | 0.20 | 0.09 | 35 | 2.08 | ***0.04*** |
| Males body weight (kg) | -1.64 | 1.43 | 35 | -1.15 | 0.26 |
| Females body weight (kg) | -1.18 | 1.23 | 35 | -0.96 | 0.34 |
| Males:females body weight | 0.82 | 0.79 | 35 | 1.03 | 0.31 |
| *Random effect* | *Variance* | *S.D* |  - | *L-ratio* | *p* |
| Males ID | 0.03 | 0.17 | 0.00 | 1 |  |
| Females ID | 0.03 | 0.17 | 0.00 | 1 |  |
| Residuals | 0.004 | 0.06 |  |  |  |
|  |
|  | *Fixed effect* | *Estimates* | *S.E* | *DF* | *t-value* | *p* |
| Offspring total length (mm) | Fertilization group | 0.08 | 0.25 | 35 | 0.31 | 0.76 |
| Males body weight (kg) | -6.28 | 3.70 | 35 | -1.69 | 0.09 |
| Females body weight (kg) | -5.46 | 3.19 | 35 | -1.71 | 0.09 |
| Males:females body weight | 2.74 | 2.05 | 35 | 1.34 | 0.19 |
| *Random effect* | *Variance* | *S.D* |  - | *L-ratio* | *p* |
| Males ID | 0.19 | 0.44 |  | 0.00 | 1 |
| Females ID | 0.19 | 0.44 |  | 0.00 | 1 |
| Residuals | 0.03 | 0.16 |  |  |  |
|  |
| Offspring body area (mm2) | *Fixed effect* | *Estimates* | *S.E* | *DF* | *t-value* | *p* |
| Fertilization group | -0.16 | 0.57 | 35 | -0.28 | 0.78 |
| Males body weight (kg) | -1.78 | 8.46 | 35 | -0.21 | 0.83 |
| Females body weight (kg) | -2.68 | 7.29 | 35 | -0.37 | 0.72 |
| Males:females body weight | -0.17 | 4.68 | 35 | -.0.04 | 0.97 |
| *Random effect* | *Variance* | *S.D* |  - | *L-ratio* | *p* |
| Males ID | 1.005 | 1.0 |  | 0.00 | 1 |
| Females ID | 1.005 | 1.0 |  | 0.00 | 1 |
| Residuals | 0.14 | 0.38 |  |  |  |