INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0684/2021/26–5–656–660
DOI: 10.17957/IJAB/15.1879
http://www.fspublishers.org

Full Length Article

Polyandrous Fertilization Enhances Offspring Survival Rate in an Indian Major Carp *Labeo rohita*

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Received 09 June 2021; Accepted 20 September 2021; Published 15 November 2021

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 to the hatchery production of familiar aquaculture candidate, the Indian major carp, *Labeo rohita*. The study found no significant differences in the rate of hatching, survival and deformity of hatchlings, standard body length, and 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 ultimately 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. © 2021 Friends Science Publishers

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 (Squires *et al.* 2012; Lewis and Pitcher 2017) and genetic benefits (Kekäläinen *et al.* 2010; Sagebakken *et al.* 2011). In many species of different taxa, polyandrous females produce eggs being higher in number, smaller in size, greater in viability and larger in yolk volume (Ward 2000; Omkar 2010; Kawazu *et al.* 2017) that ensure higher fertilization and hatching success (Jennions *et al.* 2007; Byrne and Whiting 2008). 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 have already unveiled that polyandry can enhance offspring fitness, only a limited number of studies were conducted to explore the influence of polyandry on the fitness of fish offspring (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*), one of the popular culture species in the Indian sub-continents, which was produced at 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,

To cite this paper: Rahman MM, S Kundu, SM Rahman, RT Mathew, YA Alkhamis, MS Parvez, MA Rouf, MM Rahman, M Asaduzzaman (2021). Polyandrous fertilization enhances offspring survival rate in an Indian major carp *Labeo rohita*. *Intl J Agric Biol* 26:656–660

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 losses.

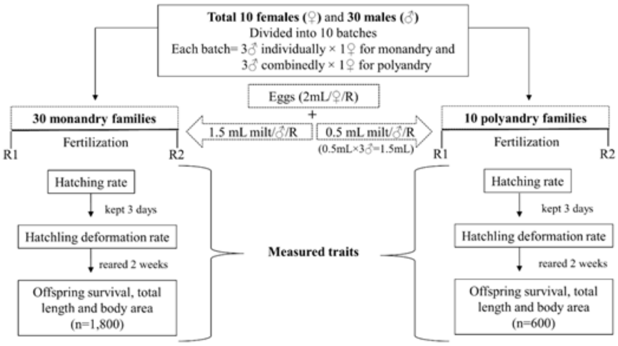

Fig. 1: Experimental design showing total number of broodstocks (*i.e.*, 30 males and 10 females), and their spawning and larval rearing processes after diving them into two fertilization groups (*e.g.*, monandry and polyandry). The entire spawning process was divided into 10 batches in which milt from three males and eggs from a single female were used during each batch. A total of 10 trials were conducted to obtain the data from 40 families. Immediately after collection, weights of total milt and eggs were measured and mixed well to have random samples. 2 mL of eggs was collected from each female with a new syringe, and simultaneously 1.5 mL milt was collected using another 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 of eggs was collected in tubes for counting and imaging later. The fertilized eggs were shifted to an assigned plastic container of 2 liter capacity, facilitated with aerated water flow, and incubated them at ambient temperature until the maximum hatching occurred. 30 larvae from each replication per family were reared for 14 days for the observation of their fitness ('n' indicates the total number of larvae per fertilization group)

Materials and Methods

Experimental approaches

Sexually mature same sized 30 males (1.59 ± 0.05 kg) and 10 females (1.33 ± 0.02 kg) were sorted up in this study to conduct a full-sib and half-sib breeding experiment (Fig. 1). Induced spawning was accomplished following the protocols of 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 hatchlings in the incubator were estimated and stocked family-wise for three days until commencing the external feeding. Then survival and details of visually deformed hatchlings were recorded from which 30 good offspring were reared family-wise in a glass aquarium (50 cm × 29 cm × 30 cm) for two weeks to assess their fitness. The pH and dissolved oxygen (DO) of water were checked daily. The offspring were fed to their apparent satiation level twice a day (Rahman *et al.* 2020). Finally, the offspring number was recorded to estimate their mortality rate. Photograph of each offspring following ice-bathed anaesthetization was taken by a digital camera for the determination of total length and body area using the *Image J* software (v. 1.46). The study was carried out up to this larval stage because most local farmers practice this system for nursing, larval rearing and marketing purposes (Rahman *et al.* 2020).

Statistical analysis

All the analyses were performed using 'R' version 3.6.3 (R Development Core Team 2020). The Shapiro-Wilk test of normality and Levene's tests for homogeneity of variance were done with 'one way tests' package. For any comparison of a measured trait between two fertilization groups, the ANOVA model was performed (using 'car' package) for normally distributed and homogenous traits, whereas Kruskal-Wallis (K-W) test was applied for traits not normally distributed by any transformation but homogenous, and the Welch test (W-T) was performed (using 'car' package) when a variable was not normally distributed as well as not homogenized.

The linear and nonlinear mixed effects (NLME) models (Pinheiro *et al.* 2019) were performed using 'nlme' package in which the 'maximum likelihood (ML)' method was followed to compare the models. In the model, fertilization group was included as a 'fixed factor' and 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 likelihood ratio test provided the *p*-values for the random effects by comparing the full model with a reduced model. 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: $F_{1,38} = 0.001$, $P = 0.99$), standard length (ANOVA: $F_{1,38} = 0.007$, $P = 0.93$) and milt weight (ANOVA: $F_{1,38} = 0.1$, $P = 0.92$) used between two fertilization groups. Similarly, common females showed no significant variations in body weight (K-W: $\chi^2 = 0$, $p = 1.0$), standard length (K-W: $\chi^2 = 0$, $P = 1.0$), egg weight (K-W: $\chi^2 = 0$, $P = 1.0$), egg number (ANOVA: $F_{1,38} = 1.34$, $P = 0.25$) and egg diameter (K-W: $\chi^2 = 0$, $P = 1.0$).

The NLME model revealed no significant variations in hatching and their deformation rate (Table 1). Interestingly, a significant difference ($t_{1,35} = 2.08$, $P < 0.05$) was found in

Table 1: Results 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			
Offspring total length (mm)	*Fixed effect*	*Estimates*	*S.E*	*DF*	*t-value*	*P*
	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 (mm^2)	*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			

offspring survival rate between these two groups (Fig. 2), while no significant variations were observed in offspring total length and body area (Table 1). The marginal effect size ($R^2m = 0.16$) of the model clearly showed the mean difference distribution between two fertilization groups with a bootstrap of 95% confidence interval (Fig. 3), which is sample size independent displaying all observed values and avoiding false dichotomy.

Discussion

In this study, the size of brood, quality and quantity of diet, and spawning procedures were maintained throughly to minimize any variation because of these factors. The experimental animal was handled cautiously to avoid any physiological stress. Moreover, the spawning procedures and random selection of the equal sized parents were 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

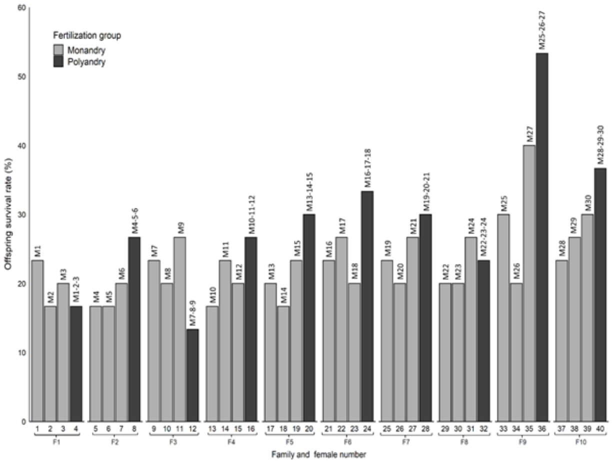

Fig. 2: The offspring survival rate (%) between two fertilization groups where 'M' (M1-M30) on the top of each bar denotes the respective male ID and 'F' (F1-F10) indicates the common female ID, while the number at the bottom of each bar is the family ID (1-40)

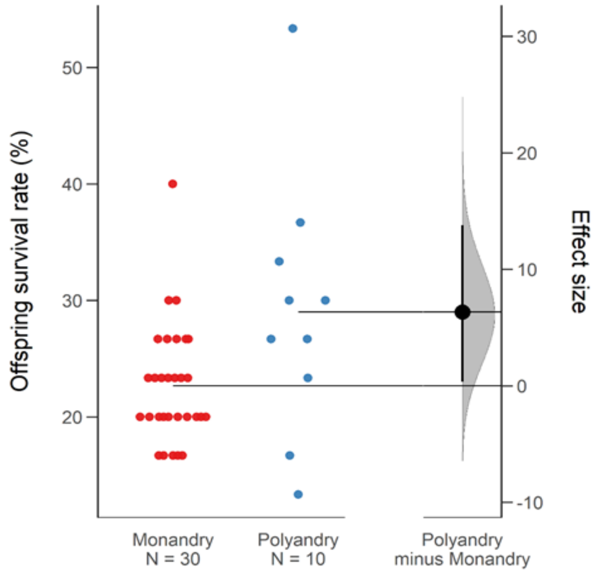

Fig. 3: The estimation plot of offspring survival rate model displaying the marginal effect size with a mean difference between two fertilization groups of *L. rohita*

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 due to the very remote location of hatchery that has very limited laboratory facilities. Moreover, sperm concentration was not possible to count because of high fat contents. At this point, total milt volume was considered only to be an indicator of male's quality following the suggestions of some previous 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 (Evans and Marshall 2005; Alonzo *et al.* 2016). 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 (Giesing *et al.* 2011; Casas and Vavouri 2014), which play important roles in offspring fitness and development. In European whitefish, offspring, fertilized from low temperature treated sperm, acquired larger body size and showed higher swimming performance 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 non-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) they prefer polyandrous rather than monandrous reproductive tactics.

Conclusion

Overall, this study provides an important information to the spawners of this species about how to obtain good quality larvae by following the polyandrous fertilization.

Acknowledgments

The authors are indebted to MH Rahman of FMRT Discipline, Khulna University for ensuring required facilities for brood stock maintenance and husbandry. This study was conducted by a grant-aid offered by Bangladesh Bureau of Educational Information & Statistics (grant no.: LS2016107), Ministry of Education, Bangladesh.

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 on the manuscript drafts.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

We hereby declare that the 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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