



Impact of sex ratio on the spawning success of zebrafish in the laboratory settings

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Abstract

Zebrafish, *Danio rerio* belonging to the family Cyprinidae is one of the most important native ornamental model species used in fisheries research globally. The present study was conducted to evaluate the effects of sex ratio on the spawning of zebrafish and to observe the ovarian maturity in the laboratory condition. This experiment was conducted for 12 weeks in the glass aquaria (25×16×26 cm³ in size) and the zebrafish were reared in five different sex ratio treatments like -T₁ (1m:1f), T₂ (1m:2f), T₃ (1m:3f), T₄ (2m:1f) and T₅ (3m:1f) and the fertilized eggs were collected daily from the aquaria. The results showed that mean number of eggs was found to be highest at T₃ (821±67.11) and the average egg lay day interval was 1.9 days which significantly (p<0.05) differ from other treatments. These research findings will provide a guideline to small scale fish farmers, entrepreneurs and hatchery owners to develop the spawning of zebrafish in the laboratory settings with a little investment. It will also provide opportunities to construct zebrafish hatchery and produce commercially zebrafish fry in a large extent.

Key words: Zebrafish, spawning, sex-ratio, spawning interval

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Introduction

The zebrafish is a tropical freshwater fish belonging to the minnow family (Cyprinidae) of order Cypriniformes (Froese and Pauly, 2007). The zebrafish is also the most popular and widely-used vertebrate model organism in scientific research, and was the first vertebrate to be cloned. As zebrafish research is to a large extent subjected on consistent production of large numbers of embryos, a solid knowledge on the breeding biology and behavior of the animal in the laboratory condition is required to ensure efficient propagation and maintenance of healthy and genetically diverse colonies. But relatively little is known about zebrafish breeding and reproductive behavior, particularly in natural settings. The factors

that affect egg production and spawning of Zebrafish in the research laboratories include the age and size of fish, interval at which fish are used for egg production, temperature, sex-ratio, light cycle, diet, and fish health status. The changes in sex-ratio may numerous enhance egg production frequency in the laboratory condition

Zebrafish are asynchronous and batch spawners, sex ratios in nature appear to be 1:1 (Spence *et al.*, 2007b) based on samples collected from a population in Bangladesh. Although zebrafish are group spawners (Spence *et al.*, 2008), both single-pair mating and group crosses are used in the laboratory (Westerfield,

1993) but there is lack of clear evidence on the effect of sex ratio in the spawning success of zebrafish in laboratory condition. So it is essential to elaborate the spawning response of zebrafish to the sex-ratio in the laboratory settings.

Zebrafish is a popular ornamental fish, frequently sold under the trade name zebra danio. The species has become a major research model used in biomedical studies to investigate, for example, vertebrate development genetics, physiology and behaviour (Grunwald and Eisen, 2002). A number of favorable attributes, including its small size, rapid development and generation time, optical transparency during early development, tractability in forward genetic screens, and genetic similarity to humans (Lamason *et al.*, 2005) have fueled its rise in popularity for biomedical studies.

As the effects of photoperiod and temperature have been broadly studied but the effects of sex-ratio on the spawning success remain largely unidentified. The present study addresses the effects of different sex-ratio on egg production. Following that the spawning frequency and ovarian maturity of the zebrafish also assessed.

Materials and Methods

Wild type adult zebra fish (*D. rerio*) were collected from the field complex pond of the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh. Good and healthy broods were selected for spawning treatments. Identification of male and female brood fish was done on the basis of some secondary sexual characters. The female zebrafish could be easily recognized by their swollen abdomen. On the other hand, the mature males were identified by their flat abdomens and slender body. Males were darker in color than females, and had more yellow coloration in the anal fin.

Total ten glass aquaria were used for this experiment each having (25X16X26 cm³) in size. The water holding capacity of each aquarium was 15 liters. All

aquaria were arranged in two rows (5 aquaria per row). Aerator was set in each aquarium. Cleaned with detergent, sponged thoroughly with tap water and siphoned out. Filled with fresh water for experimental purposes. Then fish were released into the aquaria for this experiment.

Ten glass aquaria with 15 liters' water holding capacity were used for this experiment. A total of 32 male and female fish were kept in different sex ratio in ten aquaria. Two layers of marble in a petridish were placed as a substrate and small artificial trees were also provided in each aquarium for the breeding purposes. Aerators with airstones were also provided for aeration in the aquaria. Water exchange and aquarium cleaning were done as required. Zebrafish likes clean water. For this reason, any uneaten food, dead eggs and fry, and detritus were removed from the bottom of the aquarium. This work was done daily to prevent the dirt from being accumulated on the bottom of the aquarium.

Zebrafish laid eggs every day. They eat most of their own eggs as a way of recycling the protein that is lost from producing the eggs. That is why they need high protein rich food. Fish were fed twice a day with a commercial floating feed (Mega Fish Feed Ltd.) and often with zooplankton (enriched with *Cyclops*, *Daphnia*, *Cladocerans* etc.)

Egg collection is very important for any breeding biology related study. In the present study eggs were collected at 8 AM daily from the aquarium. Eggs were deposited in between the gaps of marble in the petridish. To collect the eggs petridish was taken out of the aquarium, the eggs were separated from the marble and transferred into a hatching tray to count the eggs. Then the eggs were checked with the help of a microscope equipped with a camera (OPTICA B350, Italy) to check whether they are fertilized or not. The effect of temperature on the spawning of zebrafish was also measured during the collection and counting of eggs from each aquarium. Aquarium water temperature was also recorded using a Celsius thermometer.

Results and Discussion

Sex ratio is the ratio of males to females in a population. In the present study, the effect of different sex ratio treatments on the spawning of zebrafish was determined through the collection of eggs for about 1-12 weeks. The mean number of eggs was also varied among different sex ratio treatments. The highest mean number of eggs (821 ± 67.11) was observed at T₃ (1m:3f) and the lowest (162 ± 31.81) at T₁ (1m:1f) (Table 1). In case of spawning, there was significant difference among spawning treatments groups in the number of eggs spawned. The mean number of eggs at T₃ was significantly ($p < 0.05$) different from other treatments.

Table 1. Effects of different sex ratio treatments on egg deposition of zebrafish as per day.

Treatment	Sex ratio	Mean number of eggs per day (\pm SD)
T ₁	1m:1f	2.6 ± 1.8^a
T ₂	1m:2f	5.7 ± 1.0^a
T ₃	1m:3f	24.6 ± 1.5^b
T ₄	2m:1f	18.1 ± 1.1^{bc}
T ₅	3m:1f	15.5 ± 1.3^c

The frequency of spawning varied in different sex ratio. Here spawning frequency was measured as the mean number eggs produced at each day of a week. Egg deposition was found higher at T₃ (1m:3f) and lower in T₁ (1m:1f) (Figure 1). The highest mean number of eggs per day (24.6 ± 1.5) was observed at T₃ (1m:3f) and the lowest number (2.6 ± 1.8) was found at T₁ (1m:1f). Egg deposition at T₃ ($p < 0.05$) was significantly different than other treatments. Reproductive success in sand goby (*Pomatoschistus minutes*) were affected by changes in the sex ratio, males became less competitive for mates in a female-biased environment compared with a male-biased environment (3:6 male:female vs. 3:6 female:male sex ratio) (Kvarnemo et al., 1995). Male Guppies *Poecilia reticulata* received more responses to mating tactics (sigmoid displays, sneak attempts, and interference)

from females at a sex ratio of 2:4 compared with 1:5 female:male (Jirotkul, 1999a). In woundfin *Plagopterus argentissimus* three different sex ratios (1:1, 1:3, 1:5; female:male) did not significantly affect fertilization success (Molly et al., 2017).

This variation in results demonstrates the unique behaviors displayed by different fish species and several striking differences compared to previous studies, which may be due to differences in the fish (wild versus domesticated), environmental conditions (tank size, population density, habitat complexity), or both. Like goby, the spawning success of zebrafish was also affected by changes in sex ratio. The spawning frequency of zebrafish at different treatments were compared between 7 days and presented in the Figure 1.

The egg lay day interval was also varied among different sex ratio treatments. The highest spawning interval $4.5 (\pm 0.26)$ at T₁ and the lowest $1.9 (\pm 0.21)$ was observed at T₃ (1m:3f) (Figure 2). The interval at T₃ was significantly ($p < 0.05$) different from other treatments.

The present study confirms that sex ratio has significant effect on the spawning of zebrafish (*D. rerio*) in the laboratory settings. When the sex ratio was female biased, egg production increased significantly compared to the equal sex ratio. Earlier study proves that sex ratio bias (1:2 female:males) had no effect on zebrafish reproduction (Spence and Smith, 2005). Reproductive success in sand goby (*Pomatoschistus minutes*) were affected by changes in the sex ratio, males became less competitive for mates in a female-biased environment compared with a male-biased environment (3:6 male:female vs. 3:6 female:male sex ratio) (Kvarnemo et al., 1995). Male Guppies *Poecilia reticulata* received more responses to mating tactics (sigmoid displays, sneak attempts, and interference) from females at a sex ratio of 2:4 compared with 1:5 female:male (Jirotkul, 1999a). Sex ratios of 1:1 (female:male), 1:3 (female:male), and 1:5 (female:male) had no significant effect fertilization

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success (Webb *et al.*, 2015). In wound fin *Plagopterus argentissimus* three different sex ratio (1:1, 1:3, 1:5; female:male) did not significantly affect fertilization success (Molly *et al.*, 2017). Reproductive efficiency of zebrafish in breeding tanks in one of three sex ratios

(1 male:1 female; 3 males:1 female; 1 male:3 females) did not differ among groups, but aggression (evaluated according to presence of shed scales) was more frequently observed in the male-dominated treatment group (Ruhl *et al.* 2005).

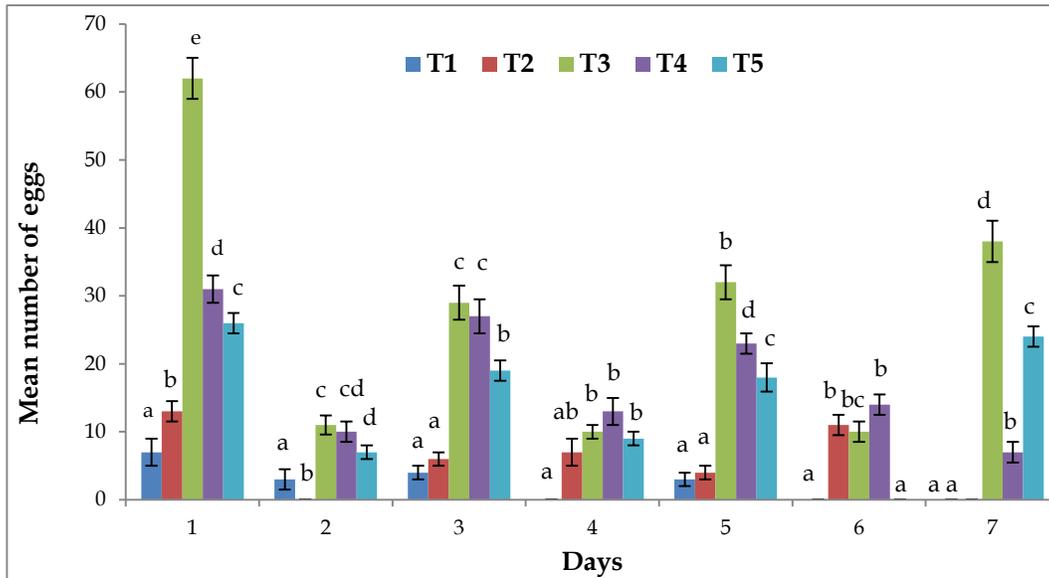


Figure 1. Frequency of spawning in different treatments in different days. subscripts of letters showed significant differences among different treatments ($p < 0.05$).

This variation in results demonstrates the unique behaviors displayed by different fish species and several striking differences compared to previous studies, which may be due to differences in the fish (wild versus domesticated), environmental conditions (tank size, population density, habitat complexity), or both. Like goby, the spawning success of zebrafish was also affected by changes in sex ratio.

The present study shows that the frequency of egg production significantly fluctuates on the daily basis. Sex ratio has significant effect on the zebrafish spawning, as eggs were collected from the female and male dominant treatment on the daily basis. On the other hand, interruption in the spawning was observed in the equal sex ratio where spawning was reduced to zero. Islam *et al* (2015) also found similar results in case of zebrafish egg production due to pH.

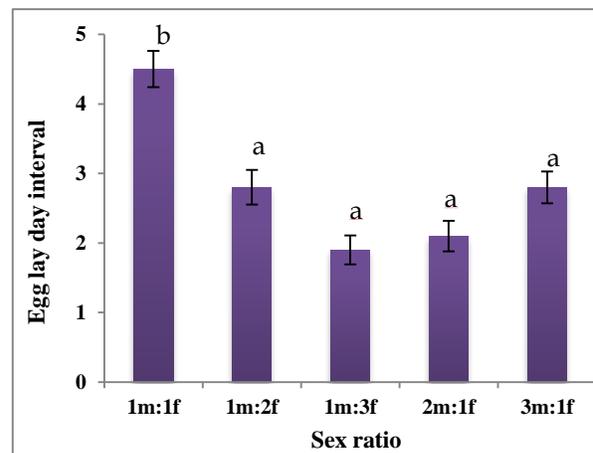


Figure 2. Frequency of sex ratio on egg lay day interval of zebrafish at different treatments. Different subscripts of letters showed significant differences among treatments ($p < 0.05$).

Another important observation is that sex ratio has also affected the interval between egg lay days. The average spawning interval was 4.5 days in T₁ (1m:3f) which is significantly higher than female dominant T₃ (1m:3f) where the average spawning interval was 1.9 days. It was previously reported that single pairs of zebrafish (*Brachydanio rerio*) 12 months old, spawned at an interval of 1.9 days when the male and female were left continuously together. Three months later the spawning interval had increased to 2.7 days, suggesting that age is a factor in determining the spawning cycle (Eaton et al. 1974b). In a study by Spence & Smith (2006) inter-spawning intervals ranged from 1 to 6 days, with a mean of 1.5 days. Like previous study on zebrafish, sex ratio was also played a worth role on the egg lay day interval. Zebrafish spawned at an interval of 1.5 days in the beginning whereas twelve weeks later the interval increased to 4.9 days. It proves that the spawning interval significantly varies with the age of zebrafish. In a study, inter-spawning intervals ranged from 1-6 days, with a mean of 1.5 days, producing clutches ranging from 1 to over 700 eggs, with a mean of 185 (Spence and Smith, 2006).

Conclusion

Zebrafish is one of the native freshwater ornamental fish of Bangladesh and spawning is an important part of zebrafish husbandry which understanding is crucial for optimizing scientific research in which *D. rerio* is used as a model organism. From the study the spawning success was found higher at T₃ (1m:3f) and at which the spawning interval was also the lowest. So, it has been proved T₃ (1m:3f) would be the best sex ratio in all aspects of zebrafish spawning in the laboratory condition. With respect to the breeding of zebrafish, this study provides clear evidence that sex ratio had significant effect on their spawning in the laboratory settings. For better production sex ratio should be carefully maintained. These research findings will provide a guideline to develop the spawning protocol of zebrafish in the laboratory settings.

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