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THE EFFECT OF LOW PH LEVEL ON ZEBRAFISH (*Danio rerio*) REPRODUCTIVE PERFORMANCE

IKRAM ISMAIL[A] AND SITI-ARIZA ARIPIN[B*]

[a,b]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Kuala Nerus, Terengganu, Malaysia*

**Corresponding author: siti.ariza@umt.edu.my*

Danio rerio or commonly known as zebrafish are a very popular fish among scientists and also a well-known vertebrate model species widely used in research. Zebrafish, are also a popular species among aquarists and have been put in aquariums all around the world as ornamental fish. The acid rain phenomenon has lowered the pH level of the wild habitat of zebrafish by shifting it to a more acidic pH level. This study was carried out to observe the effect of low pH level on the reproductive performance of zebrafish. The zebrafish were quarantined for a week to make sure they were healthy to be used in the experiment. The zebrafish were reared continuously for 14 days in three different pH treatments T1 (pH 2-4), T2 (pH 4-6), T3 (pH 6-8)). T3 (pH 6-8) was used as the control treatment. Hydrochloric acid (HCl) was used to control the pH level of treatments T1 (pH 2-4), T2 (pH 4-6), T3 (pH 6-8) with three replicates of each treatment. The male chasing female frequency was significant (p: 0.0001) and the data showed the highest frequency (2568.000±140.6272) at treatment 3 (pH 6-8). For the spawning frequency of zebrafish, treatment 3 (pH 6-8) showed the highest value (4.000±0.5774) followed by treatment 2 and treatment 1 and the data was significant (p: 0.0004). The fertilisation rate of the zebrafish was significant (p: 0.0001) and the highest was shown at T2 (pH 4-6) with 89.8018±0.3782, followed by T3 and treatment T1. For the hatching rate of the zebrafish, the data collected were significant (p: 0.0002) and the highest value of 2.9350±0.4070 was shown at T3 (pH 6-8), followed by T2 (pH 4-6) and T1 (pH 2-4). The overall result showed that pH 2-4 had the worst effect on the reproductive performance of zebrafish. Therefore, low pH has a significant effect on reducing the reproductive performance of zebrafish. The local fish population can be affected by the decrease of pH level due to acid rains and chemical waste pollution.

Keywords: zebrafish, pH, spawning frequency, fertilisation rate, hatching rate

Introduction

The zebrafish, *Danio rerio* (Hamilton, 1822), are freshwater fish native to Southeast Asia. The zebrafish are in the same family with carps and minnows and found in areas in the Himalayan region, including Myanmar, Pakistan, India, and Nepal (Kinth *et al.,* 2013). *Danio rerio* are also a good model species for vertebrate and have been used widely in research (Hoo et al., 2016). Research using *Danio rerio* have yielded advances in many fields such as biology, toxicology, reproductive studies (Hoo *et al.,* 2016), genetics, and environmental science. This species has been a good biological model system that has numerous advantages for scientists (Graham *et al.,* 2018).

The pH drops in natural habitats usually happen due to acid rains and chemical waste pollution. Diluted sulphuric or nitric acids are falling everywhere on the globe. In chemical terms, acid rain has a pH of less than 5.6 (Rogers, 2018). In some countries, the pH of acid rain can be as low as 4.2. The more acidic the water is, a lesser number of embryos can survive and hatch (Poppe, 2017). According to Poppe (2017), the number of embryos that survive and hatch decreases as the pH decreases. The release of chemical waste into the environment has also decreased the water pH in wild habitats and affected the aquatic animals and human health. In Malaysia, the pollution of chemical waste reported at Sg Kim Kim, Johor Bharu has affected the health of people and caused the death of local fish around the region (Bernama, 2019).

The changes of pH levels depend on the concentration of hydrogen ion (H^+) level in the water or aqueous solution. The decrease of pH will inhibit active sodium cation (Na^+) and increase passive Na^+ losses, leading to a decrease in plasma Na^+/H^+ level in zebrafish (Kwong et al., 2014). This is because the Na^+ uptake in freshwater fish is primarily coupled to the secretion of H^+ via the actions of Na^+/H^+ exchange and H^+-ATPase (Evans *et al.,* 2005). The role of sodium ions (Na^+) is necessary for regulation of blood and body fluids, certain metabolic functions, heart activity and transmission of nerve impulses (Fievet & Motais, 1991). When the pH decreases drastically, the zebrafish cannot maintain ammonia secretion and acid-base regulation (Das *et al.,* 2006).

For the breeding of zebrafish in a laboratory or controlled condition, the optimum conditions of water parameters are the temperature (27°C-29°C), pH (6.8-8.5), and dissolved oxygen (4-7 mg/L) (Ana *et al.,* 2016). The zebrafish can adapt well in acidic water as low as 4.0 but they might be unable to have a good reproductive performance at this extremely low pH (Kwong *et al.,* 2013). The changes of water pH in natural habitats to acidic happen due to acid rain. Acid rain has decreased fish populations in rivers and streams and according to the United States Environmental Protection Agency (EPA), most of the fish cannot hatch at a pH of 5 (Poppe, 2017). In Norway, it had been reported that the freshwater species of fish died due to acid rain (Rogers, 2018).

Danio rerio are useful as a model biological system for scientists in order to conduct experiments (Graham *et al.,* 2018). The zebrafish can be used as the model fish in the study of the effect of lower pH level on the local fish reproductive performance. The population of local fish in the wild can be estimated by testing the water pH level. To further the understanding of the influence of low pH on zebrafish reproductive performance, this study investigated the effect of pH level on reproductive performance of the zebrafish, *Danio rerio*.

Materials and methods

Animal preparation

A total of 18 adult zebrafish, *Danio rerio* with size ranging from 3.5–4.5 cm were bought at a local aquatic shop (Ming Yu Three) located at Kg Banggul Tiang Kulat, Kuala Terengganu: 9 male zebrafish and 9 female zebrafish, which were quarantined for 7 days before being used.

Experimental design

There were three treatments (T1 (pH 2-4), T2 (pH 4-6), T3 (pH 6-8)) with three replicates of each treatment. The pH was controlled by using hydrochloric acid (HCl) and the pH was dropped slowly until it reached the desired pH range for each treatment. Three tanks were used to stock water stock for each treatment. Then, the other water parameters were set up at the optimum level (temperatures (27°C–29°C), dissolved oxygen (4-7 mg/L)) and were checked using YSI Pro Plus multi-parameter and a pH metre (Ana et al., 2016; Gillespie, 2018).

Experimental trial

After the quarantine period, the fish were put into the experiment tanks (10 litres). The fish were separated according to sex for one week. The zebrafish were placed in each treatment tank with the male to female sex ratio of 1:1 (Westerfield, 2007). They were fed using commercial pallets and live feed. The water was changed around 23%–30% of the water volume every two days. After one week, the fish were placed together in a cage in each treatment. The function of the cage was to separate the broodstock and eggs. The male chasing female frequency for one hour was collected. The spawning behaviour of the zebrafish was observed (Pradhan & Olsson, 2015). After spawning occurred, the eggs sank to the bottom and the broodstock were separated from the eggs (Lawrence, 2007). The fertilisation rate and spawning rate were observed. The transparent or clear eggs were fertilised eggs while the opaque or creamy eggs were not fertilised. The hatching rate was observed and calculated after 48–96 hours of fertilisation (Zahangir *et al.*, 2015a). After 24–48 hours of hatching, the larvae were fed with live feed larvae which were *Artemia salina* (Tsang *et al.*, 2017).

Data analysis

The data were collected and analysed. All the data underwent the Shapiro-Wilk normality test. Then, the data were analysed in one-way ANOVA with SPSS using a P value of less than 0.05 to determine the significant differences between the treatment, followed by a Post-Hoc test with Tukey's test to make sure which treatment was the best (Gomez & Gomez, 1984).

Results and discussion

The data parameter values for the reproductive performance of *Danio rerio* reared in three different pH levels were significantly different for each parameter. The significant difference from the male chasing female frequency was 0.0004 and for spawning frequency, it was 0.0001. p= 0.0001 showed the fertilisation rate p= 0.0002 showed the hatching rate parameter.

Table 1: Summary of mean data parameters (±SE) of *Danio rerio* reared in different pH levels

Treatment	T1 (pH 2-4)	T2 (pH 4-6)	T3 (pH 6-8, control)	*P* value
Male chasing female frequency in 1 hour	600±27.7128[a]	1704±96.9949[b]	2568±140.6272[c]	0.0004
Spawning Frequency	0±0.0000[a]	3±0.0000[b]	4±0.5774[b]	0.0001
Fertilisation Rate (%)	0±0.0000[a]	89.8018±0.3782[c]	85.6608±0.8212[b]	0.0001
Hatching Rate (%)	0±0.0000[a]	0±0.0000[a]	2.9350±0.4070[b]	0.0002

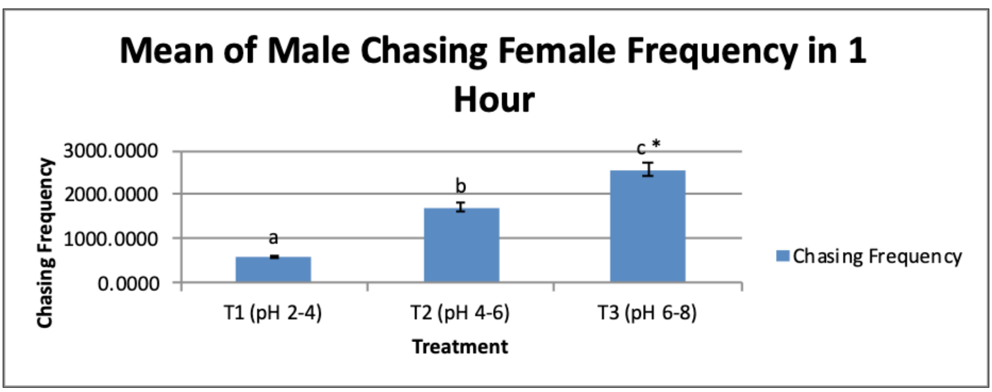

Figure 2: The mean of male chasing female frequency in 1 hour for T1 (pH 2-4), T2 (pH 4-6) and T3 (pH 6-8)

Based on the result of the mean value of male chasing female frequency in Table 1, it was statistically significant at $p= 0.0004$. T3 (pH6-8) showed the highest frequency followed by T2 (pH 4-6) and T1 (pH 2-4). According to Ana et al. (2016), for the breeding of zebrafish in the lab or controlled condition, the optimum pH level was pH 6.8-8.5. The zebrafish became more active and energetic at pH levels around neutral. Lower pH can cause chronic stress to the zebrafish. According to Zahangir *et al.* (2015b), the changes in the level of pH can cause aquatic organisms to feel stressed and get killed, even in the range of pH that can be tolerated. If the fish are stressed, they become inactive and not interested in mating. When the pH is decreased, the chasing frequency will decrease.

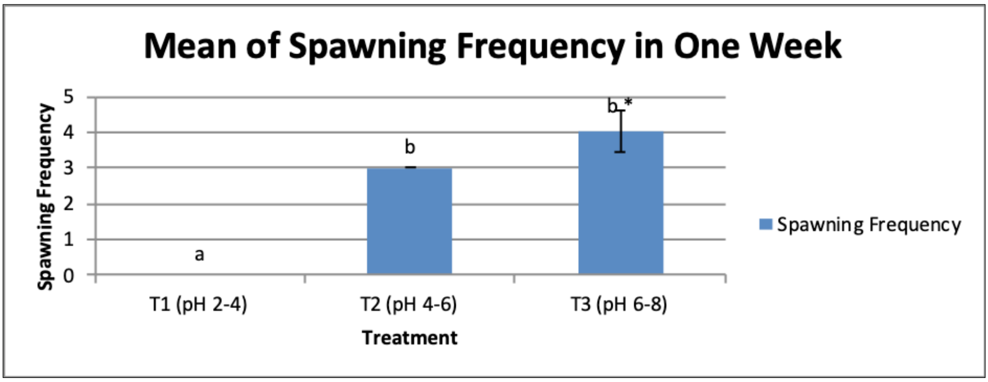

Figure 3: The mean spawning frequency of the zebrafish, *Danio rerio,* in one week at T1 (pH 2-4), T2 (pH 4-6) and T3 (pH 6-8)

The highest mean of spawning frequency in one week for the zebrafish, *Danio rerio,* was in T3 (pH 6-8), followed by T2 (pH 4-6) and T1 (pH 2-4). According to Eaton and Farley (1974), the frequency of spawning for a mature zebrafish can be two or three times in a week. A better physiological function of zebrafish was observed at a pH range of 7.0-8.0 (Lawrence, 2007). According to Lawrence (2007), the production of eggs was lower due to the secondary stress response (Prasad & Charles, 2010). Blood glucose is a secondary stress parameter that influences the maturations of gonads (Zahangir *et al.*, 2015b). When the lower pH was introduced, the zebrafish became stressed and released cortisol. The cortisol increased the blood sugar through gluconeogenesis. When the pH level was lowered, more cortisol was released which resulted in the high blood glucose change. The energy stored for the maturation of gonads decreased and as a result lowered the spawning frequency (Zahangir *et al.*, 2015b).

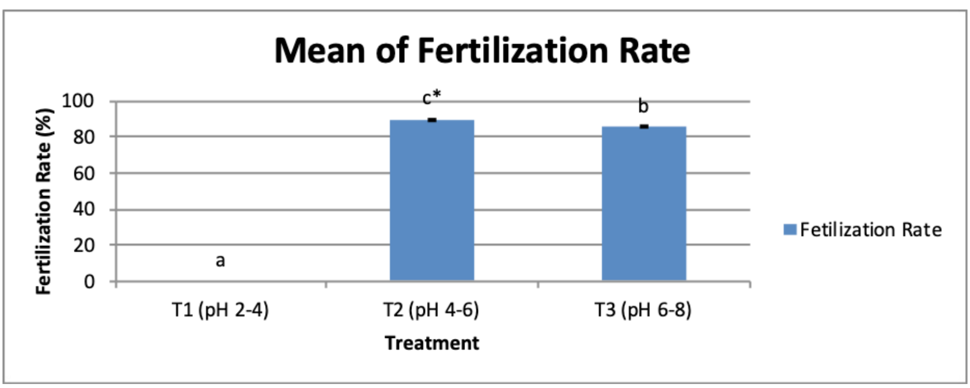

Figure 4: The mean of fertilisation rate for treatment T1 (pH 2-4), T2 (pH 4-6) and T3 (pH 6-8)

The mean of fertilisation rate for T2 (pH 4-6) showed the highest value followed by T3 (pH 4-6) and T1 (pH 2-4). Fertilisation rate was statistically significant with the highest fertilisation rate found at treatment T2 (pH 4-6). The number of eggs spawned at T2 (pH 4-6) was lower compared to the number of eggs spawned at T3 (pH 6-8). According to Bart and Dunham (1996), the largest egg mass produced the lowest fertilisation rate. The worst fertilisation treatment was shown by T1 (pH 2-4) where the pH was at a critical level for organisms to live. According to Zahangir et al. (2015a), the changes in pH may severely damage the fertilisation of embryos.

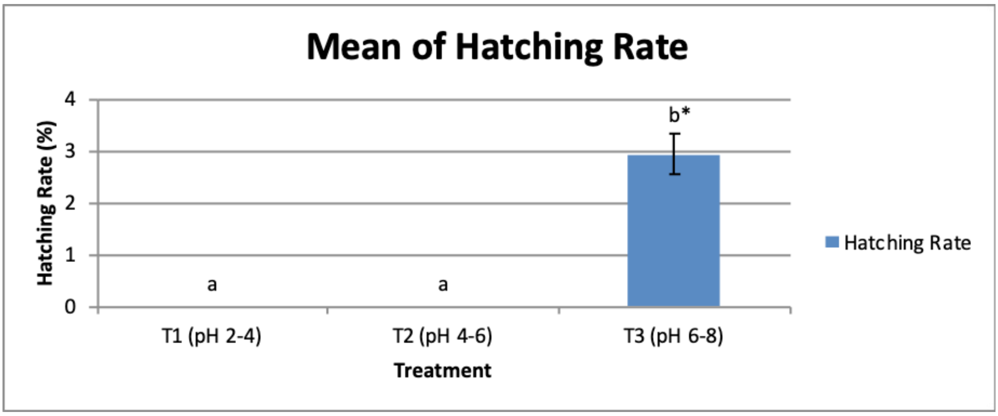

Figure 5: The mean of hatching rate (%) for the treatment (T1 (pH 2-4), T2 (pH 4-6) and T3(pH 6-8)

T3 (pH 6-8) showed the highest mean of hatching rate among the treatments with p= 0.0002. According to Poppe (2017), the number of living *Danio rerio* embryos decreased as the pH decreased from neutral (pH 7). Aquatic organisms and fish that are still in their early or immature stages are very sensitive to pH levels below 5 (Zahangir *et al.*, 2015a). According to Zahangir *et al.* (2015a), the hatching rate for zebrafish was highest at pH 7.0. In this experiment, the mean hatching rate for T3 (pH6-8) was low because there was a fungal infection on the fertilised eggs. Fungus that grow on dead or unfertilised eggs can lower the hatching rate (Gao *et al.*, 2011). The worst hatching of zebrafish was observed at T1 (pH 2-4) and T2 (pH 4-6). According to Zahangir *et al.* (2015a), the embryos were not able to undergo gastrulation at a pH range lower than 5.0. Most of the embryos died under acidic treatment and showed increasing morphological anomalies (D'Amen *et al.*, 2007).

Conclusion

In this study, it can be concluded that pH 2-4 had the worst effect on the reproductive performance of zebrafish. Acid rain and chemical waste pollution can cause the decrease of local fish population by shifting the natural water pH to acidic. Therefore, low pH has a significant effect on reducing the reproductive performance of zebrafish and local fish. The population of the local fish in the wild can be estimated by testing the pH level of the water.

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