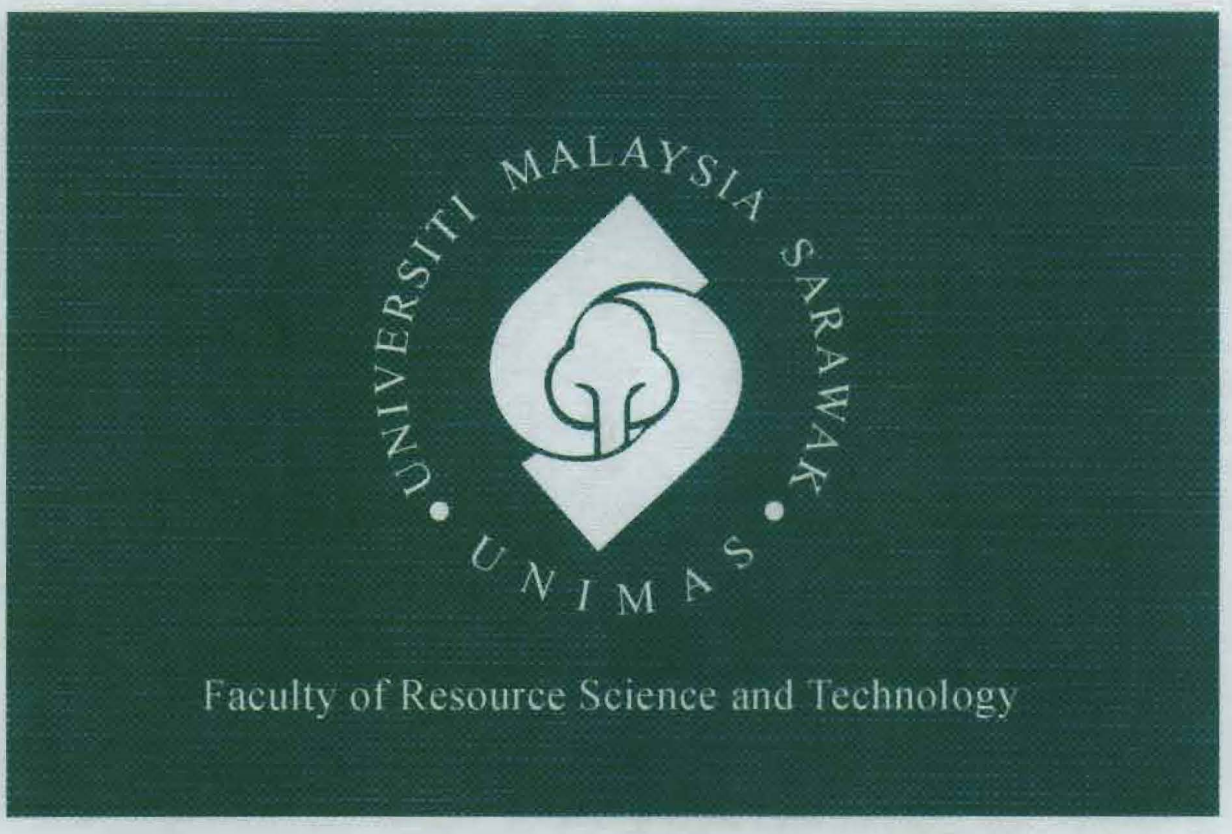

OPTIMIZATION OF ZEBRAFISH MATING SYSTEM IN UNIMAS FISH FACILITY

Jacqueline Tiong Liq Lin

Bachelor of Science with Honours
(Resource Biotechnology)
2011

UNIVERSITI MALAYSIA SARAWAK

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DUL Optimization of Zebrafish Mating System in UNIMAS Fish Facility

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Optimization of Zebrafish Mating System in UNIMAS Fish Facility

Jacqueline Tiong Liq Lin
(21099)

Final Report

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr. Lee Kui Soon

Resource Biotechnology
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ACKNOWLEDGEMENT

It is a pleasure to thank those who made this final report possible. I am deeply indebted to my project supervisor, Dr. Lee Kui Soon, for his constant support and advices. Without his help, this project would not be completed. I would also like to thank all the members of UNIMAS Animal Biotech Laboratory. I am grateful for all the support in any respect during the completion of the project.

I would like to thank my good friend Tong King Hua for his encouragements and fresh perspectives whenever I met dead-ends in planning this project. To Tie Chui Ping and Karen Lau my old friends, I owe my deepest gratitude.

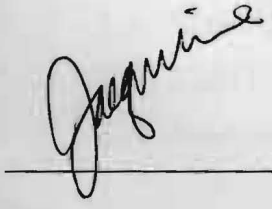
Lastly, I would like to thank my family for their support. I dedicate this Final Year Project (FYP) Final Report to my mothers and father.

DECLARATION

This final report is submitted to the Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, as part of the fulfillment of the Bachelor of Science in Resource Biotechnology.

I hereby declare that this final report submission is my own work and that, to the best of my knowledge and belief, contains no material previously published or written by another person. It has not been submitted in support of any application for another degree of qualification of this or any other university or higher learning institution.

The distribution of contribution is as such; 70% of my own contribution, 20% of my supervisor, Dr. Lee Kui Soon's input, and 10% of previous researchers on this project scope.



(Jacqueline Tiong Liq Lin)

25th May 2011

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Hpf	Hours post fertilization
Dpf	Days post fertilization

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Optimization of Zebrafish Mating System in UNIMAS Fish Facility

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ABSTRACT

Danio rerio, commonly known as zebrafish has been used as an excellent vertebrate model organism in biomedical, genetic and developmental researches. Zebrafish has advantageous characteristics of an ideal model organism, mainly its short generation time, large number of transparent embryo per mating. However, zebrafish husbandry is surprisingly under-established. Despite its usefulness in various researches, minimal researches had been carried out in the past to optimize the zebrafish's mating system. Hence, the aim of this study is to look at different parameters which may affect the fecundity of zebrafish. The parameters selected are length of light cycle, pairing of fish and water temperature. Zebrafish are selected randomly to test the hypothesis whether the mean number of viable embryo production is influenced by subjected parameters. There is no significant difference in number of viable embryo production when the mating pairs are subjected to different water temperature ($p = 0.595$). Besides that, different ratio of males to females in pairing up zebrafish does not influence the production of viable embryos ($p = 0.254$). However, there is significant increase in viable embryo production ($p = 0.000$) if the fish are exposed to longer length of light cycle. At the end of this study, UNIMAS fish facility can ensure a consistent supply of zebrafish embryos and adult stocks.

Keywords: *Danio rerio,* fecundity, embryo, mating system

ABSTRAK

Danio rerio, atau lebih dikenali sebagai 'zebrafish' digunakan sebagai model organisma vertebrata yang baik dalam bidang biomedik, genetic dan kajian perkembangan biologi. Zebrafish mempunyai ciri-ciri yang ideal terutamanya masa generasi yang pendek, bilangan embrio yang dihasilkan, dan embrio yang transparansi.Namun demikian, sistem pembiakan zebrafish tidak dikaji dengan mendalam walaupun zebrafish mempunyai banyak kegunaan dalam kajian saintifik. Maka, tujuan kajian ini adalah untuk memaksimakan produksi embrio ikan dengan memanipulasikan parameter yang dispesifikasi. Parameter tersebut adalah kitaran cahaya, suhu air akuarium, dan nisbah pasangan ikan. Ikan-ikan dipilih secara rawak untuk mengkaji hipotesis sama ada bilangan embrio ikan yang dihasilkan adalah dipengaruhi oleh parameter yang tersebut. Didapati bahawa tiada perbezaan signifikan apabila pasangan ikan diletakkan dalam suhu air yang berbeza (p= 0.595). Selain itu, nisbah pasangan ikan yang berbeza juga tidak mempengaruhi bilangan embrio yang dihasilan (p=0.254). Namun demikian, produksi embrio meningkat dengan ketara (p=0.000) apabila zebrafish didedahkan kepada kitar cahaya yang lebih panjang. Pada penghujung kajian ini, fasiliti ikan UNIMAS akan dapat membekalkan embrio zebrafish dan ikan dewasa dalam kadar yang konsisten.

Kata Kunci:Danio rerio, fecunditi,embrio, sistem pembiakan

1.0 INTRODUCTION

Zebrafish was first chosen as a model organism by George Streisinger to study the genetic basis of vertebrate neural development, based on the groundwork by neurobiologist Judith Eisen, who had initially used the zebrafish as a developmental model. It was found out that zebrafish is easier to manipulate genetically and is a better genetic system (Dodd *et al.*,2000).

Eisen (1991) credited zebrafish embryo as a model for cellular studies of neuronal development. This enabled visualization of developing neurons in real time throughout the course of embryonic development using differential interference contrast optics (Eisen, 1991). The vertebrate's embryo indeed has a greater complexity in terms of nervous system and vertebrate organ development (Dodd *et al.*, 2000).

Before zebrafish is widely used, mutational studies are conducted using *Drosophila melanogaster* or *Caenorhabditis elegans* to observe the events that induce patterning and morphogenesis of the embryo (National Institute of Health, 1998). However, invertebrate systems do not wholly reflect many aspects of the patterning and morphogenesis in vertebrate embryo although conservation in the genetic programs that determine embryo formation is present. Zebrafish, a vertebrate organism, has an advantage in this case where they are genetically more similar to humans (Lawrence *et al.*, 2005). Their biological trait and gene functions are also more similar with each other. This model system also has a completely sequenced genetic code and is well understood. Furthermore, there is numerous of well-characterized mutants available for different genetic or development studies.

Zebrafish and humans share a similar embryonic development despite the fact that adult zebrafish and humans is very different physically. This confers a huge advantage instead of using invertebrate animal models which do not share similar biological traits with human. In addition, it is important to note that scientists cannot carry out mutational researches on human; experimentation on zebrafish enables research data to be extrapolated to humans. Thus, zebrafish makes good models for human biology and diseases.

Sumanas and Lin (2004) had used zebrafish for drug target screening and validation. There are various methods identified by Sumanas and Lin to identify novel drug targets including chemical mutagenesis, insertional mutagenesis and small-molecule screens, using the zebrafish. Meanwhile, to validate potential drug targets, knockdown technology and target selected mutagenesis approaches is used.

The fish has the special characteristics which helped in experiments. The embryos develop outside its mother and are transparent. Eisen (1991) stated that the development is rapid but slow enough to watch individual developing neuron in real time. In addition, it is cheaper to maintain zebrafish as compared to maintaining mice in the laboratory.

It is advantageous to use zebrafish, mainly its ability to breed all year round, short generation time, and large number of transparent embryo per mating. However, Lawrence (2007) found that the zebrafish husbandry is poorly developed despite their usefulness in genetic and development researches. It is definitely worth it to improvise

zebrafish husbandry from time to time given that zebrafish serves such an important role in many of the current researches.

UNIMAS fish facility is established to supply zebrafish embryos and adults as experimental model for developmental studies and gene expression studies. Currently, the supply of adult zebrafish has to be obtained from external companies since there has not been any successful mating among the zebrafish in the fish facility. Thus, it is crucial to carry out this study to establish supplies of zebrafish adults and embryos for further experimentations in UNIMAS. Ultimately, the objective of this study is to optimize the zebrafish mating system by maximizing viable eggs production in UNIMAS fish facility.

In this study, the scope of experiments are breeding zebrafish by changing selected parameters, namely different pairing of males and females, length of light cycle and water temperature in which the zebrafish is housed in. The purpose of this is to find out which parameters is the most effective in producing viable embryos. It is hoped that through this project, an optimum mating system can be established since the other researches done on zebrafish husbandry are not based in Malaysia. It is not wise to directly adapt the established spawning method since the climate in our country is not the same as the findings at other location. Thus, it is crucial to carry out this study to establish supplies of zebrafish adults and embryos for further experimentations in UNIMAS.

2.0 LITERATURE REVIEW

2.1 Ecology, Distribution and Habitat

Zebrafish are endemic to South Asia (Lawrence, 2007). They are distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan. Generally, these regions have rainy and dry seasons with monsoon climate. Spence *et al.* (2007) observed that zebrafish is commonly found in shallow ponds and standing water bodies. This is consistent with Lawrence's (2007) finding that zebrafish prefer still or slow moving water, and slightly alkaline water.

2.2 General Description of Zebrafish

Kimmel *et al.* (1995) had fully described the stages for development of the zebrafish embryo; zygote period, cleavage period, blastula period, gastrula period, segmentation period, pharyngula period and hatching period. Hatching takes place between 48-72 hours at 28.5°C mainly dependent on the thickness of the chorion and the muscular activity of the embryo inside, and this varies within different group of embryos (Kimmel *et al.*, 1995)

Zebrafish mature at 10-12 weeks and can spawn every 10 days if optimum conditions are observed. Fish at the age of 6-12 months are most productive. The zebrafish breed whole year. The females lay large quantities of eggs and their eggs are fertilized externally. A pair of adult fish can lay up to 200-300 eggs in the morning. If maintenance of the zebrafish is optimum and appropriate, they can yield every 5-7 days (Hill *et al.*, 2005). They are photoperiodic breeders (Granato & Nusslein-Volhard, 1996) meaning that mating occurs within first or second hour after sunrise (Hisaoka & Firlit,

1962). Diet of zebrafish can be made up of live brine shrimp, *Artemia*, or live paramecia for baby fishes and commercial flake food. Food availability directly influences reproduction among zebrafish (Spence *et al.*, 2006).

2.3 Advantages of Zebrafish as a Model Organism

Zebrafish confers many advantages as an experiment model. The zebrafish are small in size and can be kept together in large numbers. It has short generation time that is rapid maturation of zebrafish eases mutagenesis screening, establishing transgenic lines and assessing chemicals for teratogenicity (Hill *et al.,* 2005). Besides that, embryos are robust which develops outside the mother. Furthermore, transparency of the embryo eases the staging of the development, where cells can be visualized directly in living embryo and enables *in situ* mRNA hybridization analysis in whole-mount embryos.

2.4 Breeding Method

There are two method proposed by Westerfield (2000), the simple method for steady, low-level embryo production and the method for maximal embryo production.

2.4.1 Simple Method for Steady, Low-Level Embryo Production (Westerfield, 2000)

Zebrafish produces embryos every morning, i.e. they are photoperiodic breeders. Equal number of males and females are used for continuous production of relatively lower number of embryos. The light cycle is to be kept at 14 hours light and 10 hours dark.

It is important to keep the fish well-fed with protein rich food. On breeding days, the zebrafish should be fed with dry/moist food and brine shrimp preferably at dawn. In the next hour, the fish are expected to mate and embryo can be collected. The fish would be fed lightly in the evening. For non-breeding days, zebrafish should be fed lightly with flake food to avoid them to become fat and this causes the fish to breed poorly.

2.4.2 Method for Maximal Embryo Production

This method is described by Sullivan as cited in Westerfield (2000). This method requires more labour but can produce large numbers of embryo (up to 1000 embryos per tank) once or twice a week. The males and females should be kept in separate tanks 8 females or 16 females per 10 gallon tank. The tank must be kept clean.

On the day before the embryos are wanted, the fish is fed 1-2 hours before the end of the light period and siphon out excess food. The males are transferred into the tank with the females with the ratio of 1 male: 2 females. A single layer of marbles is added to cover the entire bottom of the tank to prevent cannibalism. At the beginning of the next light cycle, embryos can be found in between marbles and is collected by siphoning. Collection of embryos from the same fish for more than 2 days in a row is avoided.

2.4.3 Raising Baby Zebrafish

The following method is described by Walker as cited in Westerfield (2000). Embryos must be kept in system water. For the first few days of fertilization, the embryos can be placed on petri dish or a 250mL beaker. The best temperature for growth and accurate staging is at 28.5 °C. The embryos normally hatch around third day of

fertilization but only need to be fed until fourth day post fertilization. The feed for larvae consists of live paramecia or other microorganism. At day 9, the larvae can be fed both paramecia and baby brine shrimp, and finally adult-type fish food. For maximum growth, keep the number of fish per tank low. Feed them at least twice daily and replace 1/3 of the water in each container daily.

2.5 Water Quality

There has not been an established water quality standard for raising zebrafish in captivity. On that matter, Sanders (2009) stated that minimal scientific consensus has been reached because very limited numbers of controlled studies have been conducted to evaluate what water quality is optimum for captive zebrafish. Housing of fish at optimum water conditions and quality will greatly influence a fish's well-being. This implies that the fishes would be stressed if they are housed outside their optimum tolerance range. The fishes would have to devote extra energy towards maintaining homeostasis for survival rather than on growth and reproduction (Lawrence, 2007).

Water quality will determine whether or not the fish would survive. There are processes like nitrogen metabolism and respiration by the fish, influences water quality directly as claimed by Buttner *et al.* (1993). Buttner *et al.* (1993) also maintains that only several factors are more significant towards the fish's well-being namely dissolved oxygen, temperature and ammonia.

All in all, water quality parameters for zebrafish cultures have to be determined to ensure fish growth and optimal environment for reproduction. Besides that, natural factors must be taken into account when setting up a mating facility for zebrafish. Presence of natural plant matter is a must to create a similar environment to that of their

natural habitat. According to Sessa *et al.* (2008), the lay rates of zebrafish can be negatively limited if the laboratory mating environments limits their natural behavior expression.

2.6 Light cycle

According to Westerfield (2000), zebrafish are "photoperiodic in their breeding" and produce eggs in the first two hours of exposure to sunlight after a period of darkness. The method for maximal embryo production in Westerfield: *The Zebrafish Book* indicates that the fishes should be exposed to 14 hours light and 10 hours darkness. On the other hand, Lawrence (2007) observed that zebrafish breed throughout the day in captivity especially during the evenings prior to an imposed dark period. This is supported by Spence (2006) cited in Lawrence (2007) which had observed zebrafish spawning during the afternoon following the onset of heavy rain.

Although it is well known that exposure to darkness and subsequently light is a necessity, limited data from previous studies had tested light cycle as a parameter to maximize eggs production in zebrafish. This study will further confirm whether or not photoperiodic does play a role in maximizing eggs production in zebrafish.

2.7 Food availability

Spence *et al.* (2006) has found out that reproduction of zebrafish is significantly affected by food availability. It was deduced that food as a necessity for life must be present, only then the zebrafish would mate. Markovich *et al.* (2007) also emphasized on the importance of food, in particular the type of food, which would affect spawning in

zebrafish. They conducted their experiment on seven-month-old zebrafish were fed four different diets to test the hypothesis that diet affects spawning success which lead to characteristics of eggs and offspring. It is discovered that zebrafish fed on trout starter bore 187.38 eggs, the highest mean number of eggs while zebrafish fed with *Artemia* bore 174.5 mean numbers of eggs. Meanwhile, the zebrafish fed on the control diet (consists of mixed *Artemia*, flake feed, and liver paste) bore 166.0 mean number of eggs. This shows that a particular type of food induces number of eggs laid.

3.0 MATERIALS AND METHODS

The experiment was carried out between 18th October 2010 and 8th April 2011. The zebrafish adults are housed in UNIMAS fish facility under the management of Dr Lee Kui Soon. The experimental system consisted of 16 glass aquaria all connected to a common water supply. Water supply (tap water) flowed through a series of filters to settle wastes, oxidizes ammonia excretion and expels chlorine in the four bottom aquaria. Water is pumped to each aquarium after the final filtration aquarium. Fish were maintained communally before experiment started.

3.1 Maintenance of zebrafish

All the fish was fed 3 times a day with fish flake food. The best method was to give no excess food than they can consume within five minutes. Excess food and debris were siphoned after the fish had stopped feeding.

Every week, the fish aquariums were cleaned and 1/3 of the water was replaced. Water temperature was kept at 26°C, room temperature at the fish facility.

3.2 Set up of Breeding Tanks

The fish were set-up in plastic tanks 8 (length) X 5 (height) X 5 (width) inches. Aquarium water was used all the time. The tanks were prepared as such: cloth meshes at the bottom of the tanks to create space for eggs to fall through and at the same time prevent the adult fish from eating their eggs (Figure 3.1 and Appendix I).

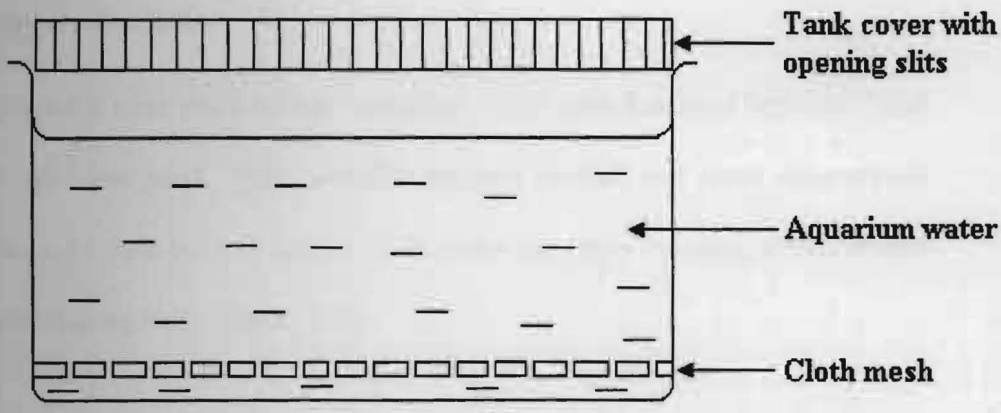

Figure 3.1: The set-up of a breeding tank

3.3 Preparation of Embryo Medium

Stock solution was prepared (10X embryo medium). The recipe for stock solution preparation is included in Appendix II. When in use, dilution was carried out to obtain 1X embryo medium from the stock solution using the following formula

$$m_1 v_1 = m_2 v_2$$

m_1 = Initial concentration

v_1 = Initial volume

m_2 = Final concentration

v_2 = Final volume

After preparing the 1X embryo medium, a few tiny drops of methylene blue is added to minimize fungus growth.

3.4 Identifying zebrafish

Zebrafish used were the wild type zebrafish. Only zebrafish aged between 7 and 18 months of age were used. Male zebrafish appears slender and more streamlined, having gold strips on their belly (Figure 3.2). Females are more rounded, differentiated by their slight protruding belly (Figure 3.3).

Figure 3.2: Adult wild type male zebrafish, *Danio Rerio (*Adopted from Lopez, 2008).

Figure 3.3: Adult wild type female zebrafish, *Danio Rerio* (Adopted from Lopez, 2008)

3.5 Procedure

3.5.1 Embryo Survival Study

Individual fish were randomly assigned to separate breeding tanks: 10 tanks contained 1 female and 1 male. As a precaution step, all the tanks was not moved or repositioned once the fish had been set-up to reduce stress on the fish. The fish tanks were left on laboratory benches in room temperature. The zebrafish were subjected to 14 hours light and 10 hours of dark (8:00 am to 10:00pm) as recommended by Westerfield (2000). An automatic timer was used to ensure the light cycle is as such.

In the next morning, the eggs were laid and fertilized within the first three hours of the light cycle. Observation was carried out to study the spawning behavior of the zebrafish pair. Mating behaviour would usually stop once they have finish laying eggs. Then, the adult fish were removed from the breeding tanks and the embryos were collected using a plastic tea strainer. The embryos were then placed in a petri dish containing 1X embryo medium.

Using a dissecting microscope (Olympus SZ51 0.8X – 4X, zoom ratio 5:1), the number of embryos collected were counted and stored with not more than 50 embryos to a petri dish. All the petri dishes containing the embryos were put in incubator at 28°C. When the embryo had reached 4 hours post fertilization (hpf) stage, the total number of viable embryos was counted again. Total number of non-viable embryo were calculated and removed from the petri dish. At 24 hpf, 72 hpf and 7 dpf the total number of viable embryos was counted respectively. The developmental stages of the embryos were determined based on their development and not based on real time (Kimmel, 1995).

After the baby fish were 1-week-old, it was assumed that all the baby zebrafish survives from that point onwards.

Survival rate at each developmental stage stated above was calculated using the formula below:-

$$\text{Survival rate} = \frac{\text{Number of Viable Eggs}}{\text{Total number of eggs produced}} \times 100\ \%$$

In this experiment, it was replicated to obtain at least 20 spawning.

3.5.2 Effect of different pairing of fish on number of viable eggs produced

Individual fish were randomly assigned to separate breeding tanks: 10 tanks contained 1 female and 1 male. As a precaution step, the tanks were not moved or repositioned once the fish had been set-up to reduce stress on the fish. The fish tanks were left on laboratory benches in room temperature. The zebrafish were subjected to 14 hours light and 10 hours of dark (8:00 am to 10:00pm) as recommended by Westerfield (2000). An automatic timer was used to ensure the light cycle is as such.

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