Artificial Reproduction of Zebrafish *Dania rerio* under Controlled Laboratory Condition

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

The objective of this study was to describe a standard universally accepted artificial culture protocol for zebrafish. Also, developmental stages of embryogenesis were also described. Because Zebrafish are considered as a model organism and are widely used in biomedical research to study human genes and human diseases. Despite this, the artificial reproduction of zebrafish is poorly described, and there is not any standard universally accepted artificial culture protocol. To satisfy the scientist working in the biomedical area, a standard culture method of zebrafish under controlled laboratory conditions must be developed.

The present study was conducted at the Aquaria Research unit of Marine Sciences and Technology Faculty of Iskenderun Technical University, Iskenderun Turkey, from March 2018 to October 2018. Spawn traps and gravels are not used for spawning of broodstock. Female and male broodstock were selected from our previously cultured stock, based on their swollen abdomens. Mean body lengths for female and male were 3.34±0.40, 2.97±0.3 cm and the mean weights were 0.38±0.14, 0.25±0.27 g, respectively. First eggs were squeezed from the ovaries then milt was taken from the testes onto the eggs. Fertilisation was done artificially by the dry method. The eggs hatch within 4-5

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days at 28±1°C. The mean diameter of eggs and fecundity were measured using a micrometre attached to a microscope. During the experimental studies, the water quality parameters were also maintained at optimum.

In the present study, temperature and pH were maintained at 28±1°C, and 7.28 respectively and which were the best for reproduction of zebrafish. Fecundity differed widely between individuals, and the number of eggs ranged from 7 to 110. The average diameter of the eggs ranged from 405± 0,001 to 570 ± 0,001 µm. Mainly seven developmental stages of embryogenesis were described; the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching stages.

The results of the present study suggest that with the right artificial culture conditions and proper manual stripping, the required number of eggs and embryos for biomedical research can be easily obtained.

1. INTRODUCTION

Zebrafish (*D. rerio*) has many advantages over the mammalian species. It has many genes in common with humans [1]. Therefore, they are considered a model organism and are widely used in research to study human genes and human diseases [2,3,4,5]. Besides their common genes with humans, they have many valuable features that are indispensable for biomedical scientists. They are inexpensive, have a very short generation, breed fast, they have high fecundity, possess a transparent body, which allows researchers to easily examine how particular genes drive myelination and to examine the effects of various compounds on the process. They distributed almost all over the world. This makes easier to obtain and produce them even in a very small aquarium. In the last decade, they have been perfect model organism for organ regeneration [6], angiogenesis [7], neuroscience [8], multiple infectious [9], stem cell development [10], and human cancer [11], It is clear that; biomedical scientist all around the world has accepted zebrafish as an important model organism.

Although zebrafish are used as model species for much biomedical research, little attention has been paid to the artificial reproduction of the species. The information on artificial reproduction is almost lacking, and there is no single standard method for zebrafish propagation. Spawn trap with aquatic vegetation and spawning over gravel are most commonly used methods [12]. In these methods, fertilisation and hatching rate is low. Spawning must be done artificially to obtain a large amount of embryo for biomedical studies. The information on spawning and the artificial reproduction of zebrafish is extremely important because it allows us to know accurately the egg number, egg size, maturation age, size and a large number of embryos at the same age which is extremely important for designing experiments. Therefore, this research is aimed at artificially to propagate zebrafish and standardise a universally accepted propagation method. Also, developmental stages of embryogenesis were investigated.

2. MATERIALS AND METHODS

2.1 Experimental Design

Facilities of the Marine Science and Technology Faculty, İskenderun Technical University, İskenderun, Turkey, were used to conduct the experiment. A static water system consisting of two acrylic tanks of 29-l capacity (23x35x37 cm) was used (Fig. 1A). The weekly water exchange was done manually and the exchange rate was 70%. The water temperature of each tank (28±1°C) was maintained by an air conditioning. The photoperiod was maintained on a 12 h light: 12 h dark schedule. *D. rerio* of known age (3 months old) originating from one pair of spawners, were stocked into each tank at the rates of five females with three males. The mean length and weight of females were measured as 3.34±0.40 cm and 0.38±0.14 g, respectively. Whereas the mean length and weight of males were measured as 2.97±0.3 cm and 0.25±0.27 g. During the experimental work, fish were fed with two different live bait feeds. First, one was artemia and the second one was daphnia. Feeding was done twice daily for a duration of six months. Fish experiments were approved by the Iskenderun Technical University in Turkey and were conducted in agreement with the guidelines of Republic of Turkey University of Iskenderun Technical laboratory animal ethics committee.

Water quality parameters were measured in 2 weeks interval. The water quality parameters were measured according to the standard
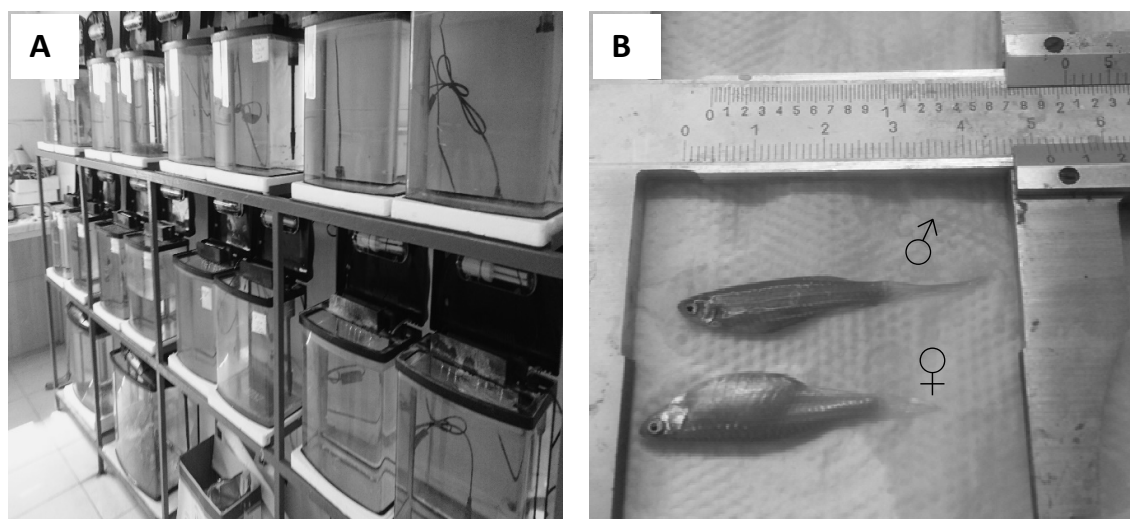
Fig. 1. A) Experimental aquariums used during the study. B) Male and female selected on the base of their morphological structure

procedure USGS (United States Geological Survey). Egg diameters were measured in 127 eggs using an ocular micrometre attached to a microscope. Stages of embryogenesis were described on the basis of Kimmel et al., [14],

3. RESULTS AND DISCUSSION

3.1 Water Quality Parameters during the Experimental Studies

During the experimental studies, water quality parameters were measured (Table 1). The temperature was maintained at 28±1°C, pH ranged between 7.06 and 7.08, and carbon concentration was 15.09-17.02 mg/L throughout the experimental period. Oxygen level varied from 4.9 to 6 mgL^{-1} Total alkalinity measured as 225- 250 mgCaCO$_3$L$^{-1.}$ The temperature was the most important parameters. When the temperature was low, stripping of males and females were not successful (Personal observation). In this study, it was vital to determine optimal ranges of water quality parameters for zebrafish cultured under controlled laboratory conditions. So that survival rate can be maximised, and fish grow rapidly and consistently produce large numbers of high-quality embryos. Data studied by Schaefer and Ryan [15] and Lawrence [12], indicate that zebrafish have a considerable thermal tolerance range of 6.7-41.7°C. In the present study, the temperature was maintained at 28±1°C which was the best for reproduction of zebrafish. Few temperature-sensitive mutants have been identified in zebrafish, because of the difficulty of rearing zebrafish at low temperature. Delomas and Dabrowski [16], developed a novel method that allowed rapid growth of larval and juvenile zebrafish at 23°C compared to previous studies in the literature. Like temperature, pH and dissolved oxygen level have profound effects on zebrafish artificial production. In the present study, the pH and dissolved oxygen level were 7.6 and 6.86 respectively. Zebrafish embryo tolerance to limited oxygen and pH level was

Table 1. Water quality parameters during the experimental studies

Water quality parameters					
Time (Months)	T(˚C) ±sd	Oxygen (mg/l) ±sd	pH (mg/l) ±sd	PO$_4$ (mg/l) ±sd	NO$_3$ (mg/l) ±sd	C (mg/l) ±sd
May	28±1	5.9±0.71	7.08±0.38	0.69±0.51	70.57±6,35	16.8±0.47
June	28±1	7.5±0.84	7.08±0.49	0.81±0.79	73.86±5.91	15.9±0.55
July	28±1	6.4±0.91	7.7±0.21	1.41±0,57	62.62±4.70	17.3±0.66
August	28±1	6.18±0.98	7.08±0.21	1.70±0.51	59.63±7.0	16.8±0.71
September	28±1	7.03±0.70	7.06±0.21	0.90±0.53	60.65±2.55	17.2±0.12
October	28±1	6.86±0.42	7.7±0.38	1.95±0.97	58.61±5.23	16.6±0.29

investigated by Andrade et al., [17], Median lethal concentrations (LC50s; 96-h) of 3.68 and 10.21 were determined for acid and alkaline pH, respectively [17]. Embryo survival appeared to be relatively resistant to oxygen depletion with a 96h-LC50 of 0.42 mg/L. However, they concluded that the concentration level of 6mg/L and below caused oedema and developmental retardations.

3.2 Fecundity and Egg Diameter of *D. rerio*

The number of eggs produced in a single spawning time (batch fecundity differed widely between individuals. The number of eggs ranged from 7 to 110 in fish between 3.34 and 2.97 cm in length and 0.38 and 0.25 g in weight (Fig 2A and B). They spawned at the 4 months of age. Their eggs were not adhesive, and they were transparent (Fig. 2C). Eaton and Farley [18], studied fecundity and egg quality. In their study batch, fecundity was 60 eggs per pair. In the current study, fecundity was much higher than those of [18] were foundlings. Eaton and Farley's study was based on their observation, and most importantly, the study was performed in natural conditions, artificial spawning was not performed. We counted the number of eggs after in vitro fertilisation in swollen, water hardened state. The results of this study indicated that the number of eggs produced in a single spawning time differed widely between individuals. It ranged from 7 to 110. Adu and Thomsen [19], obtained 40 eggs per pair. The authors also used spawning traps. In the present study, 110 eggs were produced in one pair, an improvement over the 40-60 and/or 110 eggs produced in the previous works. Our results demonstrate that with the artificial culture condition and proper optimisation of procedure

the required number of eggs and embryos needed for the biomedical research can be obtained.

The development period from fertilisation to hatching was found to be 4days at 28±1°C. The mean diameter of their eggs ranged from 405± 0,001 to 570 ± 0,001 µm. Embryos were translucent, and the optical clarity of the embryo allowed direct visualisation of individual cells and the cell movements that occur within the developing embryo (Fig. 2D).

Spawning success, fertilisation and hatching rate was quite high. The diameter of eggs were ranged from 405µm to 570µm. Mean value was 490±1,45. Cek [20], pointed out that bigger eggs or resultant offspring, have a better chance of survival than the smaller ones. Body length and body weight may be also explained because large fish may be able to produce larger eggs. Bromage and Cumaranatunga [21], concluded that small and large eggs have equal chances of survival. In the present study, the relationship between egg number, and body length, body weight and egg diameter were not investigated. However, we did not observe any differences between larger and smaller eggs in survival.

3.3 Stages of Embryogenesis in *D. rerio*

Seven periods of embryogenesis were clearly detected (Table 2). All periods occurred at 4 dpf (day post fertilisation) (Fig. 3A, B,C,D,E and F), and (Fig. 4A, and B). These developmental stages were briefly divided into seven stages. Namely: the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching stages.
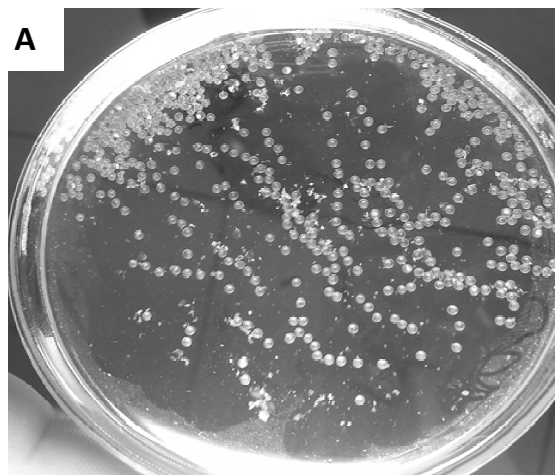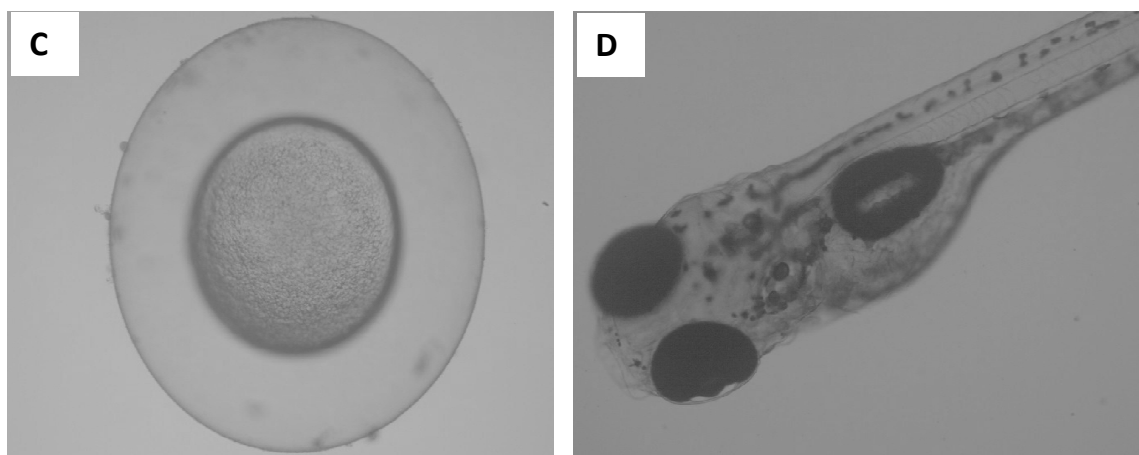
Fig.2. Eggs and embryo development in D. rerio. A) Eggs are shown after fertilization. B) Eggs can be counted easily under appreciate light. C) Recently fertilized in adhesive and transparent egg are shown. D) Translucent embryo is given

The stages of embryo development were classified on the basis of observations of changes in the morphological features of fertilised egg. During these phases, the changes were similar to those reported by Kimmel et al., [14] and Sharmili and Angelin, [22]. The only notable difference from the above studies was that the duration of the incubation period was 4 days. This difference attributed to temperature differences between this study and previous studies.

Kimmel et al., [14], studied embryonic developmental stages of zebrafish, their study was comprehensive and the developmental stages described in detail. At higher temperatures, the shorter incubation period was recorded [14,22]. The duration of the incubation period depends on water temperature in most teleost species including zebrafish [23]. In addition, changes in incubation temperature alter survival, hatchling phenotype and cost of development [23]. In an interesting study by Malek et al., [24] reducing temperature over a physiological level increases lifespan of zebrafish. However, the authors hesitated to conclude regarding the effects of decreased temperature on the lifespan of zebrafish, investigations seem promising.

In the present study, Zebrafish eggs are found to be telolecithal. The egg consists mass amount of yolk, and only a small region is free of yolk. The large yolk volume limits cell division to a small region animal pole near the micropile. These characteristics were similar to that of Kimmel et al., [14] and Sharmili and Angelin, [22].

Table 2. Stages of embryogenesis in *D. rerio* at 28±1°C

Stages	Time (Hour)	Definition
Zygote	0-15 h	A zygote is the first diploid cell. In this study, it was detected 15 minutes after fertilisation (Fig. 3A).
Cleavage	15 minutes-20 h	The yolk distribution was telolecithal which means only a small region was free of yolk (Fig. 3B)
Blastula	2-5 hpf	Somatic cell cycle detected at this stage. It was detected at 1-2hour post fertilisation (Fig. 3C).
Gastrula	5-10 hpf	At this stage, the embryonic shield was formed and made the dorsal side of the embryo (Fig. 3D).
Segmentation	10-27 hpf	At this stage, the formation of the tail was detected (Fig. 3E, F).
Pharyngula	27-50 hpf	Straightening of the spine, circulation, pigmentation, fins developments were observed (Fig. 4A).
Hatching	73- 96 h	At this stage, the formation of primary organ systems occurred. This stage was mostly detectable at the age of 4 days post fertilisation (Fig. 4B).
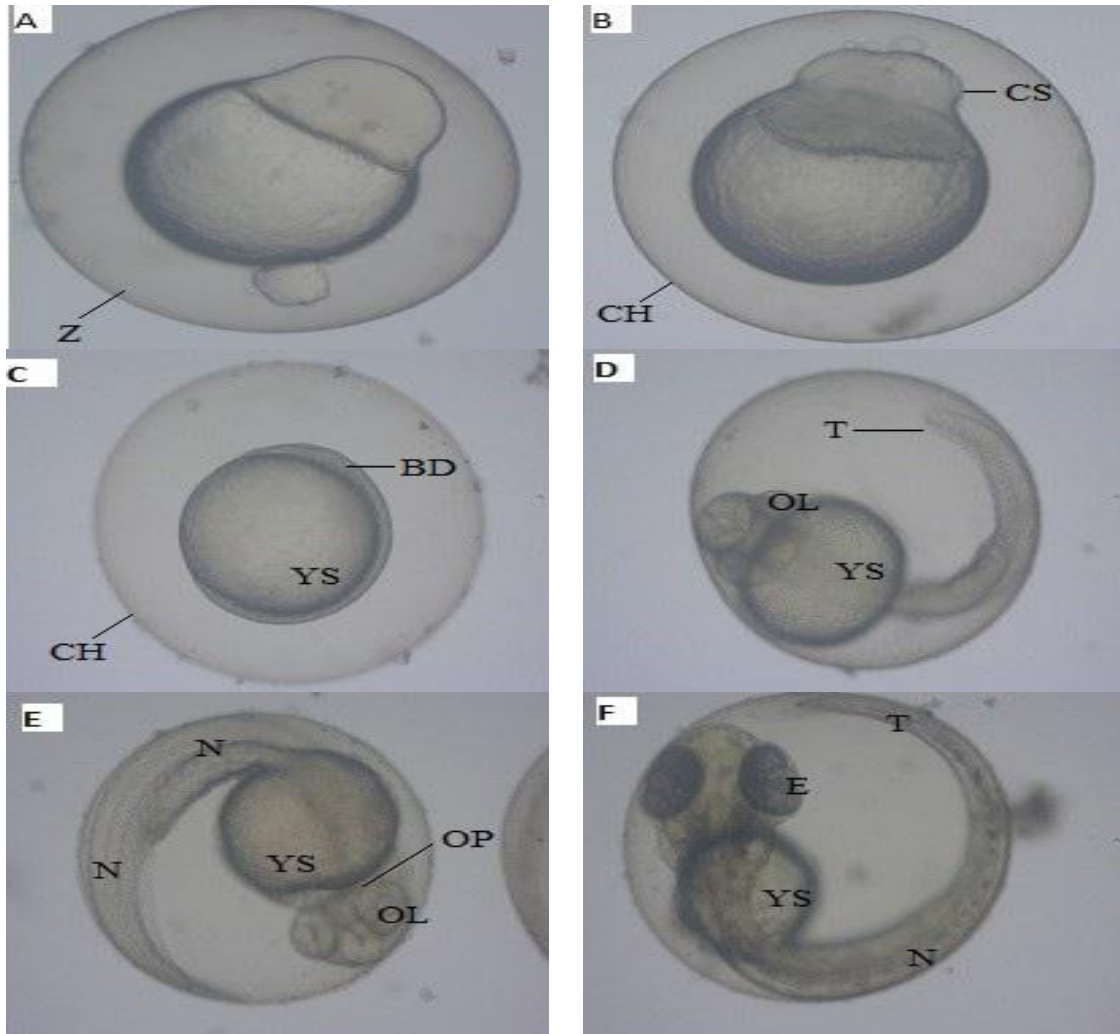
Fig. 3. Stages of embryogenesis in zebrafish. A) Embryo at the zygote stage, B) Embryo at the cleavage stage, C) Embryo at the Blastula stage, D) At the Gastrula stage, E) Embryo at the beginning of segmentation and F) Embryo at the end of segmentation stage.N, Notochord; OP, Otic Placode; E, Eyes; YS, Yolk Sac; BD, Blasto Disc; T, Tail; OL, Optical Lens; CS, Cleavage Stage; CH, Chorion
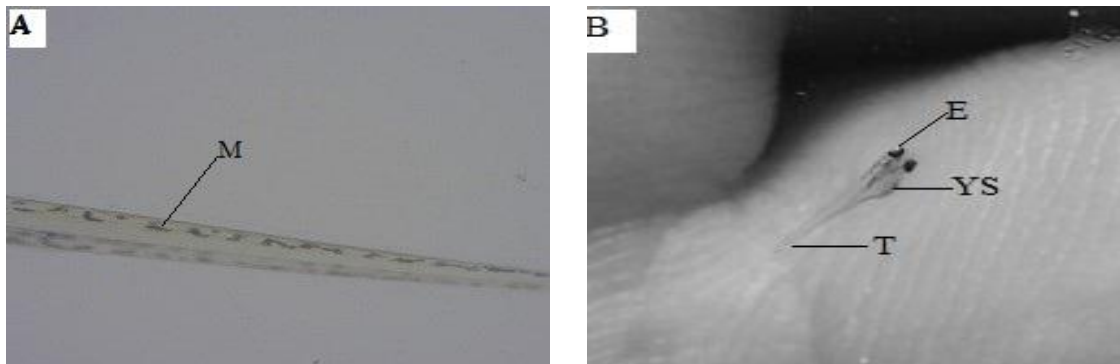
Fig. 4. Stages of embryogenesis in zebrafish. A) Embryo at the Pharyngula stage, B) Newly hatched larvae.M, Melanocytes; E, Eyes; YS, Yolk Sac; T, Tail

Historically, vertebrate developmental biology and human disease have been studied using mammalian models [25]. However, zebrafish embryogenesis has emerged as a viable alternative to these conventional models. Zebrafish model has gained popularity, particularly for developmental research. Because of its characteristics during embryogenesis. In the present study, larvae were transparent 10 days post fertilisation and major organs and tissues developed by 4 days post fertilisations. These results are valuable for developmental biology and biomedical research. Zebrafish embryogenesis has also been used for investigation of genetically caused disease [26, 27]. Davis and Katsanis [27] discussed zebrafish as a robust model system that offers similar genomic and anatomical orthology to humans. The authors compared and contrasted zebrafish to other animal model systems. Zebrafish embryogenesis has relative strengths for pathomechanism of germline mutations in humans, due to its transparency.

4. CONCLUSION

Water quality parameters particularly temperature was extremely important for the artificial propagation of zebrafish. Developmental stages of embryogenesis were briefly divided into seven stages. Namely; the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching stages. The average diameter of eggs was 490±1,45 μm. Fecundity was variable from seven to 110 eggs per individual. As a whole, these results suggest that with the appropriate artificial culture condition and proper optimisation of procedure the required number of eggs and embryos needed for the biomedical research can be obtained.

ETHICAL DISCLAIMER

Fish experiments were approved by the Iskenderun Technical University in Turkey and were conducted in agreement with the guidelines of Republic of Turkey University of Iskenderun Technical laboratory animal ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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