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Zebrafish breeding in laboratory environment at TIFR

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Abstract

Laboratory zebrafish (*Danio rerio*) has proved promising vertebrate animal model in modern biology research during past few decades. Several inherent biological features in zebrafish, have invited attention of the scientific community to use them in genetics and developmental biology research. Attempts were made to establish facility to raise and maintain several lines of wild type and mutant zebrafish at the Department of Biological Sciences in 2009. A small room in the department housed ~50 wildtype zebrafish obtained from the ornamental fish shop at the local market (M/s. Vikrant Aquaculture, Mumbai). At the beginning, fish were raised in glass aquarium of 50 liters capacity for 6 to 12 months. During the period of initial setup, all the members of the group associated with the usage of zebrafish,received training in the zebrafish husbandry, water quality control, fish breeding and health monitoring. The state of the art facility was established later on by setting Recirculating Water System (RWS) procured from the Aquatic Habitat, USA. The wild type (WT) and mutant fish lines procured from the Max Plank Institute (MPI), Germany,were kept in RWS after ensuring healthy aquatic environment for the fish. The standard operating protocols for fish husbandry, breeding, nutrition, water and health quality of zebrafish were formulated.The breeding parameters such as fecundity, egg spawning, viability and mortality were studied. The female fecundity percentage noticed between 42 to 58 and the mean egg spawn found between 139.77 to 242.42 eggs per female in five strains. In-vitro culture of embryos revealed maximum mortality on first day of culture with decreased mortality on subsequent days. Mortality percentage observed during the period of culture ranged between 14.35 to 30.21 in five strains with mean mortality of 21.34%. The embryo viability in five strains ranged between 69.78% to 85.64% with mean viability of 78.65%. In the present study, attempts have been made to generate basic data associated with breeding of the laboratory zebrafish.

Key words: zebrafish, breeding, spawn, embryo, larva

Introduction

The laboratory zebrafish (*Danio rerio*) as a research model of human biology is more and more evident each year. The invaluable biological characteristics inherited in zebrafish, promise it as a model organism for studying genetic mechanisms of vertebrate development and diseases. Its rapid embryonic development, transparency of its embryos and the large number of offspring make it ideal for discovering and understanding the genes that regulate embryonic development as well as the physiology of the adult organism. The species has become a major research model used in biomedical studies to investigate vertebrate development, genetics, physiology, and behavior (Grunwald and Eisen, 2002). Zebrafish is a freshwater fish that were originally found in slow streams, paddy fields and in the Ganges River in East India and Burma (Fig-1). Zebrafish is available now a days at pet store throughout the world as an ornamental fish. Considering their potential use as a model organism in biomedical research, several WT and mutant lines generated in the laboratory and started rearing in the aquaria by providing optimum conditions necessary for their survival and breeding. With the increasing usage of laboratory zebrafish in the areas of biomedical research, drug testing and toxicity studies, many research laboratories, drug testing industries and educational institutions have come up with zebrafish facilities. With the larger scope in research, training and education, a comprehensive understanding of the husbandry of zebrafish is essential to ensure efficient propagation and maintenance of healthy and genetically diverse colonies.

Materials and methods

Aquaria:

The department has state of the art zebrafish facility with continuous recirculating water, procured from the Aquatic Habitat, USA. The system has capacity of holding 240 (10L), 720 (3L) and 400 (1.5L) aquaria with stocking density of 30,000 adult zebrafish. Larval, baby and adult stages of wild type and mutant lines of the laboratory zebrafish were housed in RWS. The system is equipped with inbuilt provision of water heater, oxygen aeration and degassing, UV sterilization, biological, mechanical and chemical filters. The sterilized water is supplied in the fish aquaria continuously after filtration through biological, mechanical and chemical filters. Mechanical filters remove course and fine debris such as uneaten feed and fish fecal matter in the aquaria. Chemical filters neutralize highly toxic chlorine and organic chemicals in the fish water. Biological filters harbor *Nitrosomonas* and *Nitrobacter* species of the nitrifying bacteria necessary for the conversion of highly toxic ammonia and nitrite into less toxic nitrate. Each individual aquarium supplied with sterile water without contaminating water of the adjacent aquaria, ensuring no water mixing among all the aquaria.

Water quality:

The WT and mutant lines of zebrafish were raised using municipal corporation water in four stand-alone systems (SAS, Fig-2) and reverse osmosis (RO) water in centralized zebrafish facility (CZF, Fig-3). The corporation water stored in ~ 400 L capacity water tank after passing though domestic Aqua guard water filters was supplied to SAS units. The corporation water desalted using RIOS-100 (Merck Millipore) and mixed with red sea salts (25 g/L) and sodium bicarbonate (16 g/L) to adjust conductivity and pH respectively was supplied to CZF system. Initially, the fish aquaria water was conditioned by adding few fish in the aquaria on regular interval to enrich nitrifying bacteria on biological filters in the system. The quality of water was regularly monitored for water parameters using digital thermometer (temperature), pH and conductivity meter (dissolved oxygen, conductivity and pH), water analysis kits, Merck (chlorine, nitrite, nitrate and ammonia) to keep the levels in the suitable range (Table-1). The temperature of the fish aquaria water was maintained between 27^0C to 29^0C using inbuilt water heaters in the system. Embryos and young larvae have special requirement and were raised in egg water.

Photoperiod:

Zebrafish are photoperiodic in breeding and spawn eggs soon after sunrise in natural conditions. In aquarium environment, maintenance of constant light and dark period in the room especially for the breeding fish is essential. The facility was illuminated using fluorescent lights with light intensity between 100 to 150 lux, regulated by timer to provide constant light (13 hrs) and dark (11 hrs) cycle.

Zebrafish lines:

All the stages of WT and mutant line of laboratory zebrafish were maintained at the zebrafish facility of the department. Long fins line having 3-4 mix phenotypes was procured from local ornament fish shop (M/s: Vikrant Aqua Culture, Mumbai) initially to begin the facility. DBS line was derived from longs fins having typical silver and blue lines with short fins. Albino and Tubingen are well characterized WT strains and were procured from MPI, Germany. Few mutant lines maintained at the facility were procured from foreign collaborators and were propagated by setting breeding crosses and established their colonies.

Fish Nutrition:

The laboratory zebrafish were fed with highly nutritious and balanced diet. The diet feeding protocol in the facility was standardized. All the fish were provided enough diet with frequent small feedings rather than a single large feeding. All the fish were fed as per the stage of the zebrafish determined by the age of the fish. Four different types of diets were provided at four timings to achieve proper growth and maintain breeding potential of the fish (Table-2). Different types of feed such as larval diet, brine shrimp cyst, baby food and adult diet are available in the market. Adult fish were fed twice a day, however multiple light feeding that allow the fish better opportunity to utilize the food were supplied to larval and baby stages.

Zebrafish breeding:

More than 3 months old sexually matured and healthy stock of the zebrafish was used to set breeding crosses (one male and one female) in static water using breeding cages. As per the standard operating protocols of the facility, the breeder animals were given one week rest before setting next cross. The breeding cages consist of one big and other small tank. The smaller tank has perforated bottom. The smaller tank can be easily fit into the bigger one allowing embryos to descend through perforated bottom and accumulate into bigger tank. The design of breeding cage provides physical separation between eggs and fish, thus avoiding consumption of their own eggs. In laboratory conditions, maintenance of constant light and dark cycle is essential for breeding animals. The females usually lay eggs next morning soon after the onset of light cycle, if the constant light and dark photo period is maintained. The breeder fish were paired 1-2 hours before the end of light period and allow them to remain overnight in breeding cages. Following morning, breeding cages were examined for the clutch of embryos. The embryos were collected by siphoning them from the bottom of the tank and transferred into 90 mm petri dish containing E3 medium (60X Buffer: 5mM NaCl-172g, 0.17mM KCI-7.6g, 0.33mM $CaCl_2.2H_2O$-29g, 0.33mM $MgSO_4.7H_2O$-49g in 10L MQ water; 1X Buffer: 160ml, 60X buffer, 30ml (0.01%) methylene blue in 10L MQ water). The embryos spawn from each pair were observed under stereomicroscope, counted for the numbers and recorded.

Data from 50 females each of the four WT (Long fins, DBS, Albino and Tubingen) and one group of mixed mutant lines (Cla:GFP+,Cld:GFP-, Penner, NSO42) crossed for colony propagation over a period of 3-4 years was collected for the present study. The number of females that spawned the eggs and number of eggs produced per female were used for the study (Table-3).

Embryo bleaching:

In order to minimize the risk of pathogen transmission within a fish population,surface disinfection (bleaching) of embryos is common practice at many fish laboratories. The embryo bleaching protocol standardized at the MPI was used for this purpose (Michael *et al.,* 2002). The embryos were bleached using sodium hypochlorite solution (4-6%, Merck) at the concentration of 800 ul per liter of water before 30 hours post fertilization (hpf). After removing dead and abnormal embryos, the live embryos were collected in plastic tea strainer. The embryos were given bath in bleach solution and normal water for 5 minutes each followed by transfer into sterile 90 mm petri plate. The petri plate containing E3 buffer mixed with 10 ul Pronase (30 mg/ml) incubated at 28.5 ^{0}C temperature in BOD incubator.

Embryo culture:

The embryos were cleaned by removing debris and washing with E3 buffer for 2-3 times. For optimal growth, 60 embryos were cultured in 90 mm petri plates containing E3 buffer at 28.5^{0}C temperature in BOD incubator. Embryos were monitored daily for the normal development and removed abnormal or dead embryos from the culture plates. The number of dead embryos in culture plates was counted for 5 consecutive days and recorded (Table-4). The viable embryos were also recorded for analysis (Table-5).

Larval culture:

The larvae generally hatch out by rupturing chorionic membrane between 48 to 72 hpf. The larvae with well-developed swim bladder were transferred into 3L capacity aquaria on 6th day post fertilization (dpf). The young larvae have special requirements and need to be raised in conditioned water. Deionized water mixed with red sea salt at the final concentration of 3 gm/L was used for larval culture. Sixty larvae were raised in 3L capacity aquaria with 1.5L conditioned water till 12th dpf in static water. The yolk in zebrafish larvae depleted after 6th dpf and need external feeding. The larvae between 6th to 12th dpf were fed as per the feeding scheduled followed at the facility (Table-2). After 12th dpf, larvae were supplied with system water at the rate of 25-30 drops per minute and increased water flow as the age advanced. After 30 dpf, the larvae were decongested in the original aquaria and adjusted 20-25 baby larvae/3L acquaria for further developmemnt till the age of 4 months.

Results

Breeding:

The breeding data collected from 50 breeder females each from four WT and one mixed mutant lines used to cross for egg spawning were analyzed for the study. In long fins strain, total 22 females spawned 3572 eggs from 50 females used for crosses. The percentage female spawn was 44 with mean egg production per female was 162.36. Twenty one females spawned total 2935 eggs from 50 pairs in Albino strain. Forty two percent female spawned eggs with an average 139.77 eggs per female. From 50 DBS breeder females used for crosses, 29 females spawned 4346 eggs. Fifty eight percent female spawned with average production of 149.86 eggs per female. In Tubingen strain, 23 females spawned 4381 eggs with average production of 190.48 eggs per female. Forty six percent female were spawned eggs. Data collected randomly from 50 different mutants females showed 28 females spawned 6788 eggs. Fifty six percent mutant females spawned with an average production of 242.42 eggs per female. In all the five strains 123 breeder females (49.2%) spawned 22022 eggs with an average 179.04 egg per female (Table-3).

Embryo culture:

A total of 32,327 embryos obtained from the four WT and one mutant (mixed lines) were cultured *in-vitro* in E3 buffer over a period of time. A total of 4571, 4510, 11691, 5717 and 5838 embryos obtained from long fins, DBS, Albino, Tubingen and mutants were cultured for propagation of respective fish lines. Total number of dead embryo/larvae in long fins, DBS, Albino, Tubingen and mutants noticed over a period of five days were 656, 787, 3533, 977 and 948 with 14.35, 17.45, 30.21, 17.08 and 16.33 percent mortality. The large numbers of dead embryos were noticed on day first of culture in all the five strains (433, 721, 2517, 691 and 546) with 9.47, 15.99, 21.52, 12.8 and 9.35 percent mortality. The mortality percent was reduced from 2nd day of culture onwards and was negligible on 5th day. The lowest (546, 9.35%) and highest (2517, 21.52%) mortality on 1st day of culture was observed in mutants and Albino strains respectively. However, the overall lowest (656, 14.35%) and highest (3533, 30.21%) mortality during five days culture period was noticed in long fins and Albino strains respectively (Table-4). Simultaneously, the embryo viability in all the five strains were also analyzed and found to be highest (3915, 85.64%) and lowest (8158, 69.78) in long fins and Albino strains respectively (Table-5).

Discussion

In the present study, breeding parameters such as female fecundity, egg spawning, mortality and viability of embryos/larvae in laboratory zebrafish (*Danio rerio*) reared at the zebrafish facility of TIFR were studied. The data collected from 50 mating crosses each of four WT and one mutant (4 nos.) lines of zebrafish over a period of 3-4 years were used for analysis. Female fecundity in long fins, DBS, Albino, Tubingen and mutant found was 44% (22/50), 58% (29/50), 42% (21/50), 46% (23/50) and 56% (28/50) respectively. Female fecundity percent evidenced between 42% and 58% in five zebrafish strains.In DBS strain, 58 percent (29/50) of female breeders spawned the eggs, whereas 42 percent (21/50) females of Albino line spawned eggs. Five strains of the zebrafish exhibited an average egg spawning between 139.77 to 242.42 eggs per female. After combining together eggs spawned by all the five strains, the mean egg spawning was 179.04 per female. In mutant strains, the mean eggs spawning (242.42) per female was highest when compared with all four WT strains. In WT strains, the lower mean egg production could be associated with the in breeding factor used to propagate the line. In mutants, the higher mean egg spawning could be possible due to heterogeneity resulted from

outcrossing and intercrossing. These results indicated normal spawning behavior of zebrafish females which range between 70 to 300 eggs per female supported by earlier report (Michael *et al.,* 2002). In pair-wise crossings (one male and one female) spawning success of 50% is typical. One can expect to collect up to 100 fertilized eggs, but individual fecundity can be as high 350 (David white, 2005). The environmental factors such as diet, temperature, photoperiod, water quality along with age and health of the breeder animals could affect the spawning performance. Mate choice and mating behavior depend on olfactory cues, visual stimuli and social interactions. Spawning is affected by the age and size of fish, interval at which fish are used for egg production, light cycle, diet, and fish health status (Andrzej and Matthew,2012).

In their experiments, Markovich *et al.,* (2007) noticed that eggs spawning were affected by different diets in zebrafish. Fish fed with flake diet produced significantly fewer eggs (mean, 116) than fish fed all other diets (mean, 166-187). Reproductive maturity and spawning efficiency also depend on the size of the fish (Eaton and Farley, 1974). Zebrafish reproduction depends strongly on photoperiod. Mating is initiated at the onset of light, and spawning typically takes place over a short period thereafter (Breder *et al.,* 1966; Westerfield, 1993; Spence *et al.,* 2007). It has also been reported that fertilization rates are affected by not only the percentage but also the types of fatty acids in the diet (Meinelt *et al.*, 1999).

In-vitro culture of total 32,327 embryos from four WT and one mutant (mixed lines) over a period of time witnessed maximum mortality on first day of culture which reduced subsequently from 2nd day onwards. After five days of culture the total mortality percentage noticed includes 14.35, 17.45, 30.21, 17.08 and 16.33, in long fins, DBS, Albino, Tubingen and mutant strains. The lowest (546, 9.35%) and highest (2517, 21.52%) mortality on 1st day of culture was observed in mutants and Albino strains respectively. However, the overall lowest (656, 14.35%) and highest (3533, 30.21%) mortality during five days culture period was noticed in long fins and Albino strains respectively (Table-4). Simultaneously the data for embryo/larvae viability during in-vitro culture was also analyzed in five strains. The highest (3915, 85.64%) and lowest viability (8158, 69.78) was noticed in long fins and Albino strains respectively (Table-5). Earlier reports supported the present findings noticed by us. Decreased fertility and viability are often associated with inbreeding depression (Mrakovcic and Haley 1978, 1979). Degenerated eggs laid by females as well as unfertilized eggs could lead to mortality during early development. However, eggs produced by small fish show higher mortality rates and are of lower quality than the eggs derived from large individuals (Uusi-Heikkila *et al.,* 2010).

The differences in the female fecundity and eggs spawning performance among five strains could be associated with strains specific performance. Breeding is a complex process influenced by a number of factors which play crucial role in zebrafish husbandry. Widespread use of *Danio rerio* in research, training and education requires a comprehensive understanding of the husbandry of this species for efficient propagation and maintenance of healthy and genetically diverse colonies. Investigation of *D. rerio* reproduction in the wild as well as in the laboratory setting is of high importance for husbandry.

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Table-1: Aquarium water parameters monitored at the zebrafish facility, TIFR

Sr. No.	Parameters	Normal Range	Sr. N.	Parameters	Normal Range
1	Temperature (^{0}C)	27 to 29	6	CO_2 (mg/L)	<20
2	pH	6.8 to 7.8	7	Chlorine	Nil
3	Conductivity (uS)	300 to 400	8	NH_3 (mg/L)	<0.1
4	Hardness (mg/L)	100 to 300	9	Nitrite (mg/L)	< 0.1
5	Dissolved O_2 (mg/L)	6	10	Nitrate (mg/L)	< 50

Table-2: Diet feeding timings and diet types provided at the zebrafish facility, TIFR

Age Group	Four feeding times			
	9.00 hrs	12.00 hrs	16.00 hrs	19.00 hrs
6 to 12 dpf	Larval Diet	Larval Diet	Larval Diet	Larval Diet
13 to 60 dpf	Live Artemia	Live Artemia	Live Artemia	Live Artemia
61 to 120 dpf	Live Artemia	Baby Diet	Live Artemia	Live Artemia
Above 121 dpf	Live Artemia	**No Diet**	Adult Diet	**No Diet**

Table-3: Strain wise breeding data showing total number of breeding pairs crossed, number of females spawned eggs, number of eggs spawned, % of female spawned and mean egg spawn per female.

Sr. No.	Strain	Total BP	Total BP	Total eggs	% BP	Average egg
		Paired	Spawn	Spawned	Spawn	spawn/female
1	Long fins	50	22	3572	44	162.36
2	DBS	50	29	4346	58	149.86
3	Albino	50	21	2935	42	139.77
4	Tubingen	50	23	4381	46	190.48
5	Mutants	50	28	6788	56	242.42
		250	**123**	**22022**	**49.2**	**179.04**

Table-4: Strain wise culture of embryos, dead embryo noticed on five consecutive days with daily percent mortality and total and percent mortality.

Sr. No.	Zebrafish Strain	Cultured embryos	Mortality noticed during 1st to 5th day					Total mortality (%)
			Day 1, Nos. (%)	Day 2, Nos. (%)	Day 3, Nos. (%)	Day 4, Nos. (%)	Day 5, Nos. (%)	
1	Long fins	4571	433 (9.47)	70 (1.53)	105 (2.29)	26 (0.57)	22 (0.48)	**656 (14.35)**
2	DBS	4510	721 (15.99)	35 (0.77)	8 (0.18)	21 (.047)	2 (0.044)	**787 (17.45)**
3	Albino	11691	2517 (21.52)	848 (7.25)	74 (0.63)	51 (0.45)	43 (0.36)	**3533 (30.21)**
4	Tubingen	5717	691 (12.08)	74 (1.29)	117 (2.04)	62 (1.08)	33 (0.57)	**977 (17.08)**
5	Mutants	5838	546 (9.35)	13 (0.22)	240 (4.11)	135 (2.31)	14 (0.23)	**948 (16.23)**
	Total	**32327**	**4908 (15.88)**	**1040 (3.21)**	**544 (1.68)**	**295 (0.91)**	**114 (0.35)**	**6901 (21.34)**

Table-5: Strain wise culture of embryos, total mortality and viability with percentage

Sr. No.	Strains	Total embryos cultured	Total mortality (%)	Total viability
1	Long fins	4571	656 (14.35)	3915 (85.6
2	DBS	4510	787 (17.45)	3723 (82.5
3	Albino	11691	3533 (30.21)	8158 (69.7
4	Tubingen	5717	977 (17.08)	4740 (82.9
5	Mutants	5838	948 (16.23)	4890 (83.7
		32327	**6901 (21.34)**	**25426 (78.**

Fig-1: Male (upper) and female (below) zebrafish (*Danio rerio*). Source: Google images.

Fig-2: Stand Alone System for raising WT and Mu
at the department of Biological Sciences, TIFR, M

Fig-3: Centralized Zebrafish Facility (CZF) established at CML-NCRA, Pune