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Propagation of old and non-productive zebrafish lines using IVF technique

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ABSTRACT

The laboratory zebrafish (*Danio rerio*) is a well-established vertebrate animal model in frontline areas of modern biology research, including screening of therapeutic molecules and assessment of toxic chemicals. High fecundity is one of the important reproductive traits in zebrafish females that benefit researchers in obtaining a large number of embryos for high throughput experiments. External fertilization offers easy access to the embryos up to 5 days post fertilization without ethical concerns whilst optical transparency of developing embryos allows for imaging of the entire embryo, which is not practical in mammalian models. Despite being prolific breeders, laboratory zebrafish exhibit a reduction in reproductive performance after two years. In vitro fertilization, which uses frozen sperm, is an effective method for reviving genetically modified fish lines. In this study, we attempted to us e an IVF approach to re store zebrafish lines from aged zebrafish. A total of 12 zebrafish lines were reproduced using the IVF procedure, including aged fish that are unable to spawn naturally. A total of 974 (66.96%) and 1438 (55.67%) embryos survived utilizing the IVF procedure with germ cells from both mutant and wild-type parents, respectively. Zebrafish embryos survived at a rate of 67.34% in the 2–3-years age group and 55.48% in the 3-4 years age group.

Keywords: Zebrafish, CWR, mutant, IVF,

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INTRODUCTION

The research community has witnessed exponential growth in the usage of laboratory zebrafish (Danio rerio) as an animal model in the advancement of biomedical research and assessing the toxicity during the past few decades (Veldman M and Lin S, 2008; Choi EK, et al., 2023; 3. Ben-Zvi I, et al., 2023). The useful features including a fully sequenced genome, sturdiness to survive in suboptimum conditions, high level of fecundity in females, transparent embryos, and visible fertilization & development outside of the mother's body have popularized laboratory zebrafish among the research community (Teame T, et al., 2019). Having prominent advantages over other species, zebrafish has become a promising research model in biomedical

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studies to investigate vertebrate development, genetic analysis, cancer biology, infection biology, regenerative biology, behavioural analysis, etc. (Teame T, et al., 2019). With the ever-increasing usage of zebrafish in several areas of biomedical research, the research community also encountered health issues in zebrafish. Given the fact that healthy animals are paramount in obtaining reliable and reproducible research outcomes, zebrafish harbouring sub-clinical diseases can have a serious impact on the research (Kent, et al., 2012). With the larger scope of research, training, and education, a comprehensive understanding of the husbandry of zebrafish is essential to ensure the efficient propagation and maintenance of healthy and genetically diverse colonies (Kohale KN, 2021). The breeder zebrafish with poor reproductive performance can have a direct impact on the research activities including microinjection, drug screening and chemical toxicity due to the non-availability of enough embryos. In vitro fertilization (IVF) is a quite useful technique for various purposes including obtaining next-generation progeny of endangered animals, animals with infertility, animals with low or non-productive performance, using frozen germplasms and also studying fertilization mechanisms (Purohit GN, 2020).

MATERIALS AND METHODS

Aquaria: The wild type and mutant lines of zebrafish were maintained in a state-of-the-art zebrafish facility operated on the principle of continuous water recirculation (Aquatic Habitat, USA) established at the Department of Biological Sciences, TIFR, Mumbai. The aquatic housing system is designed to hold approximately 1500 aquaria of 1.5 Litre, 3 Litre, and 10 Litre capacity with a total stocking capacity of ~45-50K adult zebrafish. The aquatic system is equipped with an inbuilt provision of biological, mechanical, and chemical filters, water heaters, water chiller, aeration, degassing, and UV sterilization. The UV-sterilized water is circulated in the fish aquaria continuously after filtration through a series of filters (biological, mechanical and chemical). The housing system provides high water exchange (5-30%) over 24 hours to keep the water healthy for the housed zebrafish.

Water quality: Millipore (REOS-100) water purification system installed in the facility is used to treat municipal corporation water. To maintain the conductivity and pH of the system water, red sea salts (25gm/L) and sodium bicarbonate (16gm/L) solutions are being used by the system, respectively. The water parameters were regularly monitored for temperature, pH, conductivity, and dissolved oxygen using integrated pH/conductivity meter. The fish housing system has inbuilt biological filters that convert highly toxic nitrogenous compounds (ammonia) into less toxic (nitrate) agent. The nitrogenous compounds such as ammonia, nitrite and nitrate were measured manually using kits (Merck).

Water temperature: The temperature of system water is maintained between 27 to 29^{0}C at all times using inbuilt water heaters and chiller in continuous water recirculation (CWR) system. In the zebrafish housing system, temperature is maintained at 28^{O}C.

Photoperiod: Zebrafish are photoperiodic in breeding and spawn eggs soon after sunrise under natural conditions. To mimic the natural environment the fish aquaria is maintained with constant light: dark cycle (13:11 hrs) with illuminated white lights of intensity between 150 to 300 lux unit.

Fish Nutrition: Zebrafish of all age groups were fed with a highly nutritious and balanced diet. Four types of diets (larval diet, brine shrimp cyst, micro pellet and adult diet) were provided at four different timings (at 9.0, 12.0, 16.0 and 19.0 'O' Clock) to achieve proper growth and maintain them in healthy conditions.

Identification of fish lines: The zebrafish mutant lines that were not spawning by natural mating were selected for the IVF experiments. The breeder fish used for the study were divided into two age groups, i.e., 2-3 years and 3-4 years. A total of 26 breeding pairs (BP) which includes 13 BPs (2-3 years) and 13 BPs (3-4 years) were used for the IVF experiments. The Wild Type (WT) and Mutant breeder fish were segregated and fed with live artemia (INVE, USA) in the morning and adult diet (Zeigler, USA) in the evening.

Setting the crosses: The old and non-productive male and female mutant animals were paired, one hour after the last feeding in the evening **(Fig.1).** Simultaneously, young sexually matured male and female wild-type fish were paired. The animals were paired in breeding tanks on a 1:1 basis overnight and males were separated from females immediately after the onset of lights in the next morning. The males and females were collected in separate tanks until they are used for germplasm collection.

Fig 1. Setting the cross (1:1) of the breeder zebrafish one hour after the evening feeding.

Stripping males for sperm: To collect sperm, the wild type (WT) or mutant (Mut) males were anesthetized using 0.02-0.04 % MS222 solution. As per the protocol of Matthews JL and Varga ZM, 2021, anesthetized males were rinsed with isotonic PBS solution and excess water was removed by rolling on a paper towel. The males were fixed in the slit made on a wet sponge with the ventral side up to observe the urogenital pore under a stereomicroscope. The urogenital opening was located and then the abdomen was gently palpated using a forceps. A milky colour sperm fluid that is visible at the urogenital opening was collected using a glass capillary fitted with a rubber tube using mouth suction **(Fig. 2).** The clear watery fluid is urine and should be discarded. The milky sperm fluid was transferred to a 0.5 ml centrifuge tube having 100 µl sperm solution (SS300) and stored at room temperature. The anesthetized males were placed in a recovery bath containing system water and returned to their original tanks after the recovery from anesthesia.

Squeezing females for eggs: To collect eggs, WT or mutant females were anesthetized using 0.02-0.04 % MS222 solution. According to the protocol of Matthews JL and Varga ZM, 2021, anaesthetized females were rinsed with PBS solution and surplus water was removed by rolling the fish on a paper towel. Females are then placed on the left or right side of a 60 mm petri dish **(Fig. 3).** The fingers were soaked with isotonic PBS, and the fish was held with the thumb and first finger of one hand. Using the other hand, gently press down on the abdomen (anterior to the poster) with a finger or a little spatula to extract the eggs from the females. Egg clutches were removed from the abdomen using a small paintbrush soaked in isotonic PBS solution. The anesthetized females were placed in a recovery bath containing system water and returned to their original tanks after recovery from the anaesthesia. The good quality eggs appear golden colour, and granular, with little fluid and no intermixing of white colour eggs. The eggs that are opaque and watery were discarded.

Fig 2. Stripping the male zebrafish for the collection of sperms, A. Fixing the anesthetized male in slit on a wet sponge with ventral side up. B. Gentle palpation of belly with forceps in left hand and aspiration of sperm with a capillary attached to mouth tubing in right hand under a stere omicroscope, C. Transfer of stripped sperm into a microcentrifuge tube.

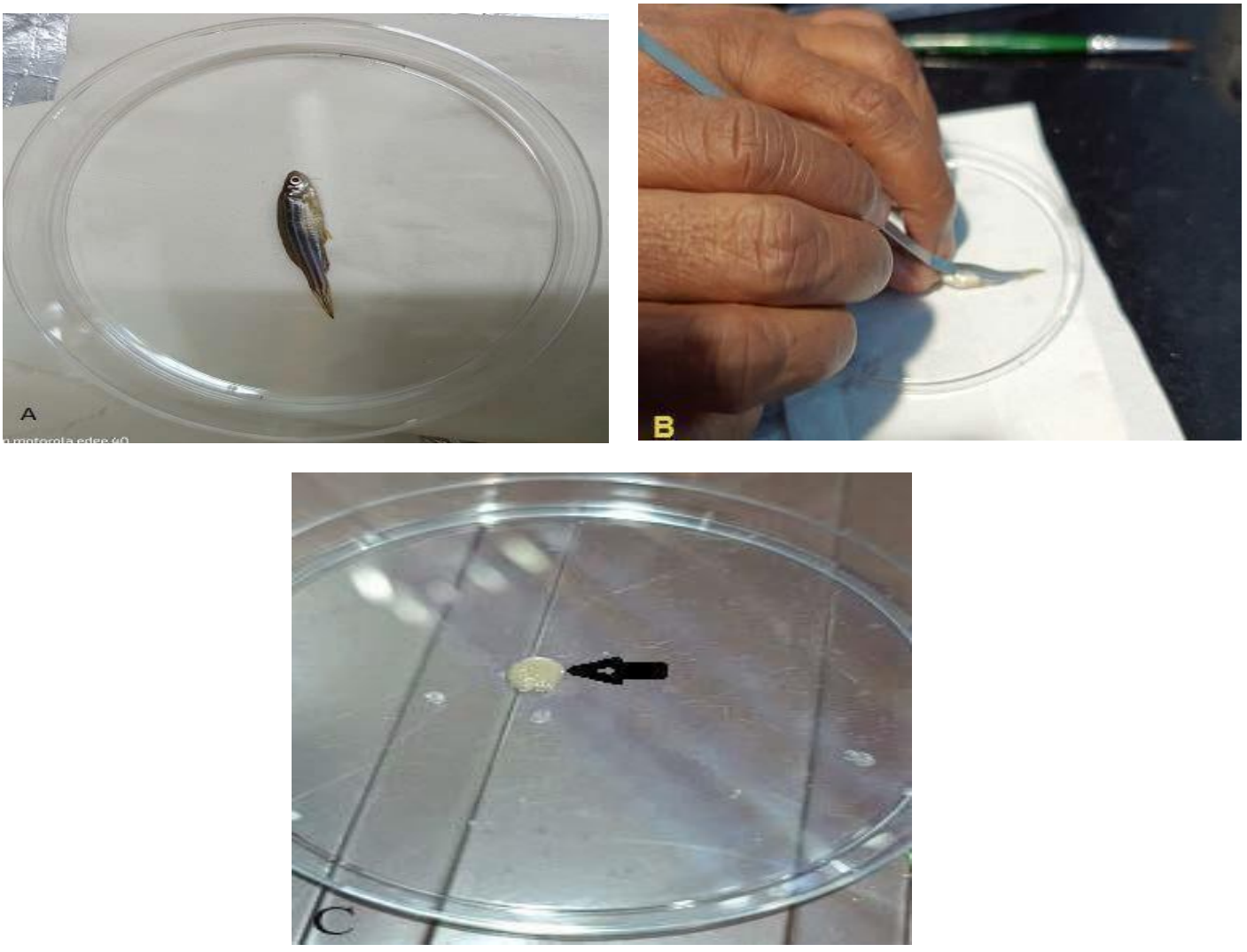

Fig 3. Squeezing of the female zebrafish for obtaining eggs, A. Anesthetized female zebrafish on lateral recumbancy kept on 60 mm plastic petri disc, B. Holding female's head in between thumb and first finger of one hand with gentle pressing of the belly with small spatula in other hand, C. Clutch of freshly sqeezed eggs (Arrow). .

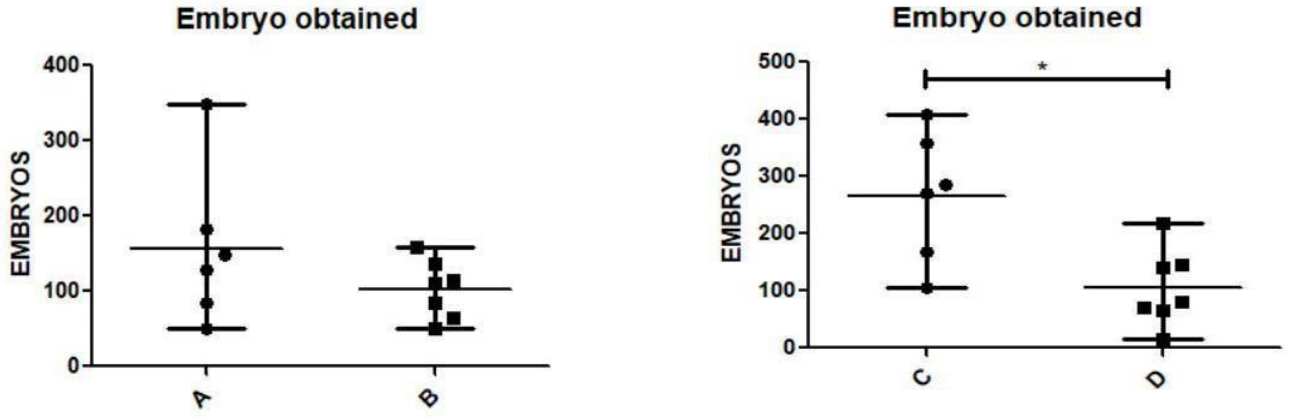

Fig. 4. a. Mean embryos (156.83±42.43) obtained from WT X Mut parents (A column) verses mean embryos (102.71±14.74) obtained from Mut X Mut parents (B column) in 2-3 years age group. b. Mean embryos (265.83±46.30) obtained from WT X Mut parents (C column) verses mean embryos (105.28±22.33) obtain ed from Mut X Mut parents (D column) in 3-4 years age group.

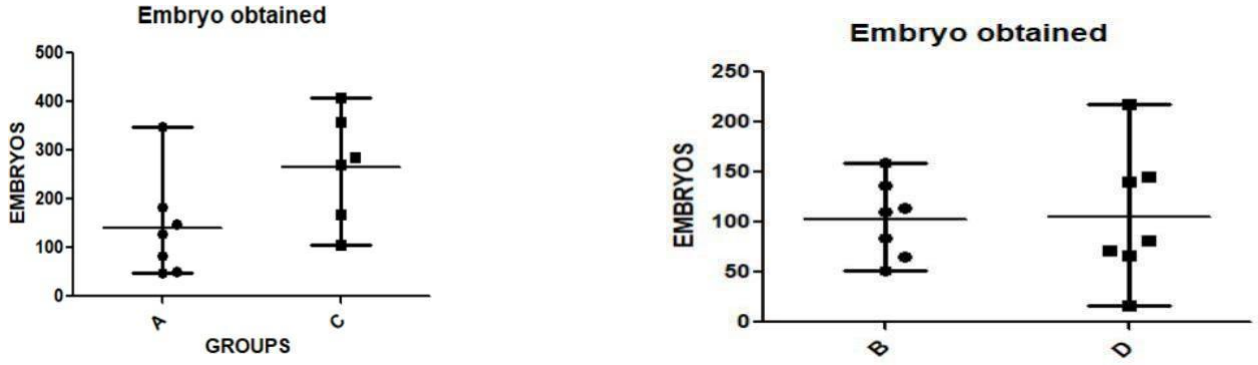

Fig. 4. c. Mean embryo obtained (156.83±42.43) in 2-3 years age group (column A) verses mean embryos obtained (265.83±46.30) in 3-4 years age group (column C) from WT X Mut parents. (P<0.05). d. Mean embryo obtained (102.71±14.74) in 2-3 years age group (column B) verses mean embryos obtained (105.28±22.33) in 3-4 years age group (column D) from Mut X Mut parents.

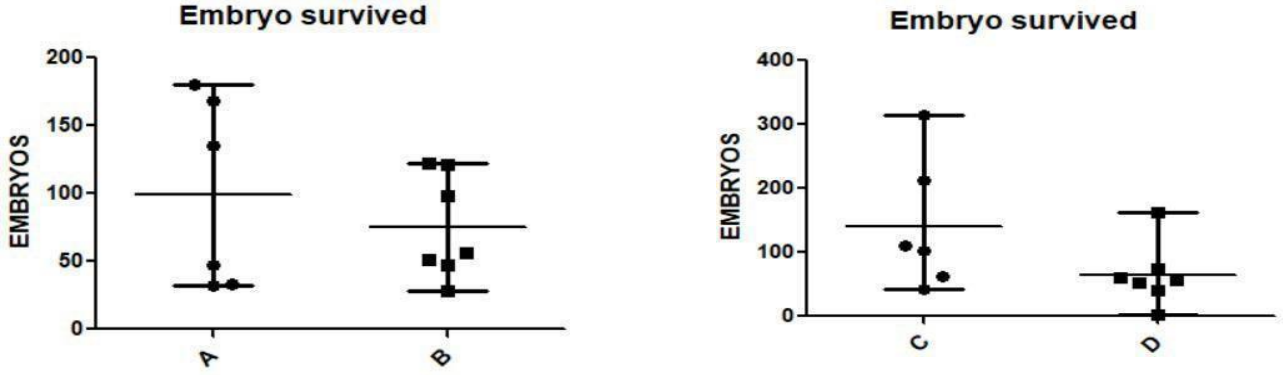

Fig. 5. a. Mean embryos (99.16±28.38) survived from WT X Mut parents (A column) verses mean embryos (74.71±13.39) survived from Mut X Mut parents (B column) in 2-3 years age group. b. Mean embryos (140.5±42.36) survived from WT X Mut parents (C column) verses mean embryos (64.42±16.85) survived from Mut X Mut parents (D column) in 3-4 years age group.

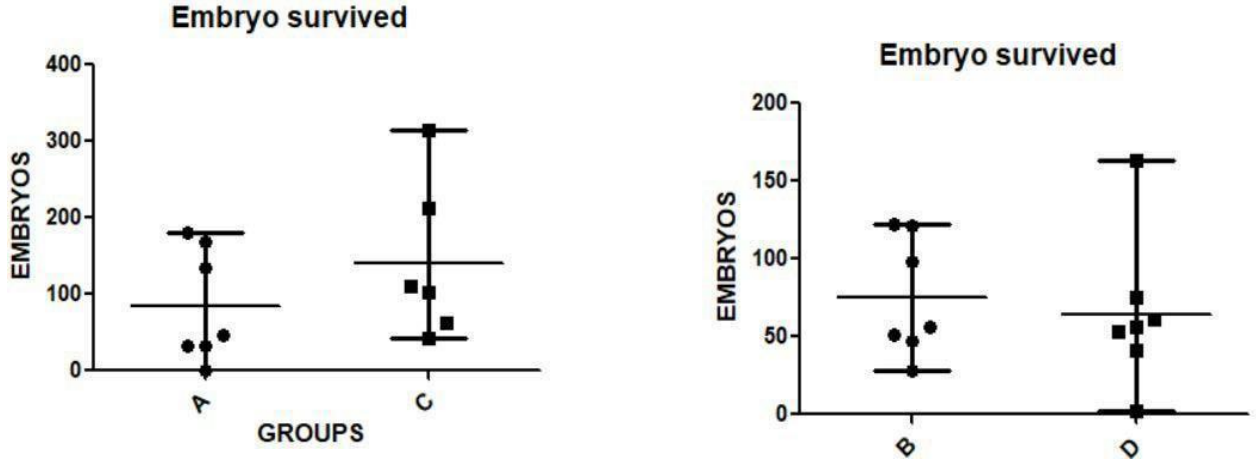

Fig. 5.c. Mean embryo (99.16±28.38) survived in 2-3 years age group (column A) verses mean embryos (140.5±42.36) survived in 3-4 years age group (column C) from WT X Mut parents. d. Mean embryo (74.71±13.39) survived in 2 -3 years age group (column B) verses mean embryos (64.42±16.85) survived in 3 -4 years age group (column D) from Mut X Mut parents.

In-vitro-fertilization (IVF): The sperm from the WT male was used for IVF with eggs obtained from mutant females and vice versa. Similarly, the sperm and eggs collected from the mutant breeders were used for IVF. The sperm fluid was mixed with SS300 solution and was transferred to egg clutches that were collected in the 60 mm Petri dishes as per the protocol. The pipet tip was slid along the bottom of the petri dish into the pile of eggs and expelled the activated sperm into the clutch of eggs as per the protocol of Matthews JL and Varga ZM, 2021. For the activation of sperms, a few drops of E3 buffer were poured on the mixture after 10 seconds and flood petri dish with E3 buffer after 2 minutes. The sperm and egg mixture were incubated at 28.5^{0}C temperature. The embryos were observed after 3 hours for normal developing stages. The dead, unfertilized embryos were removed and the live embryos were counted.

Statistical analysis: The data was analysed by Mann Whitney test using GraphPad Prism 5 Software. GraphPad Software 225 Franklin Street. Fl. 26 Boston, MA 02110 USA.

RESULTS

A total of twenty-six IVF experiments were carried out using parents from mutant (Mut) and wild type (WT) lines, aged between 2-4 years for the stripping of sperms and squeezing of the eggs**.** Among twenty-six IVF experiments, fourteen experiments were carried out using sperm and eggs from mutant parents, whereas twelve experiments were carried out using sperm and eggs from wild-type and mutant parents reciprocally. Using the IVF technique total of 974 embryos (66.89%) survived from the 1456 embryos obtained using germ cells from the mutant parents (**Table 1**) in both age groups. A total of 1438 (56.70%) embryos survived from 2536 embryos obtained through IVF carried out using germ cells from mutant and WT parents **(Table 2)** in both age groups. The mutant and WT parents used for the IVF experiments were also divided into two groups as per age (2-3 years and 3-4 years). In the first age group (2-3 years) 523 (72.73%) of the 719 embryos and 595 (63.23%) of the 941 embryos obtained from the IVF between mutant/mutant and WT/mutant parents respectively survived. In the second age group (3-4 years) 451 (61.19%) of the 737 embryos and 843 (52.85%) of the 1595 embryos obtained from the IVF between mutant/mutant and WT/mutant parents respectively were survived. The average embryos obtained from the Mut/Mut parents were 102.71±14.74 and 105.28± 22.33 in the age group of 2-3 and 3-4 years respectively whereas the average embryos obtained from the WT/Mut parents were 156.83±42.43 and 265.83±46.30 in the age group of 2-3 and 3-4 years respectively **(Table 3, Fig. 4 abcd)**. The average embryos that survived from the Mut/Mut parents were 74.71±13.39 and 64.42±16.85 in the age group of 2-3 and 3-4 years respectively whereas the average embryos survived from the WT/Mut parents were 99.16±28.38 and 140.5± 42.36 in the age group of 2-3 and 3-4 years respectively **(Table 4, Fig. 5abcd)**. There was no significant difference observed in the embryo production when the data was compared between the age groups (2-3 years and 3-4 years) for WT/Mut parents, (2-3 years) for WT/Mut and Mut/Mut parents and (2-3 years and 3-4 years) for Mut/Mut parents. However, a significant difference ($P<0.05$) in embryo production was noticed when data was compared between WT/Mut and Mut/Mut parents in the age group of 3-4 years **(Table 3, Fig.4b)**. No significant difference was noticed in the survival of the embryo when data compared among the age groups and parents. In the majority of aged zebrafish males, the quality of the sperm was good which resulted in fertilization of the eggs; however, degenerated and watery eggs were obtained from the majority of aged females.

DISCUSSION

The mutant (Mut) and wild type (WT) parents aged between 2-4 years, produced 3992 embryos using the IVF technique. A total of 2412 (60.42%) of 3992 embryos obtained through the IVF technique were viable and raised for the next generation propagation. No significant difference was noticed in the production and survival of embryos irrespective of age and parents except in one age group of 3-4 years when the embryo production data was compared between WT/Mut and Mut/Mut parents. The average embryo production and embryo survival ranged between 102.71-105.28 and 64.42-74.71 embryos respectively for Mut/Mut parents whereas 165.83-265.83 and 99.16-140.5 embryos respectively for the WT/Mut parents. The aged adult breeders used in the present study did not yield enough embryos even after repeated mating however, with the aid of IVF technique the old zebrafish lines were propagated and retained the line from the loss due to non-productive performance. The zebrafish females show high levels of fecundity with more than 80% fertile embryos (Hoo JY, et.al., 2016), however, as the breeder becomes old, the breeding potential is reduced. In the majority of zebrafish facilities, the common practice is to terminate the breeding stock after 2 years of age, however, in certain experiments, the breeder zebrafish needs to be maintained for the ongoing experiments even if the age is advanced. The various factors such as advanced age, inbreeding, disease, or experimentally induced health issues like ENU-induced or targeted mutations are accountable for spawning decline in zebrafish (Castranova D and Wang

Table 1: Embryos obtained and survived from mutant parents using IVF technique.

Sample No	Age of the parents (Yrs)	Embryo obtained	Embryo survived	Age of the parents (Yrs)	Embryo obtained	Embryo survived
1	2-3	110	51	3-4	140	53
2	2-3	84	28	3-4	145	41
3	2-3	159	122	3-4	81	75
4	2-3	65	56	3-4	66	56
5	2-3	51	47	3-4	71	61
6	2-3	114	98	3-4	16	2
7	2-3	136	121	3-4	218	163
	Total	**719**	**523**		**737**	**451**

Table 2: Embryos obtained and survived from wild-type and mutant parents using IVF technique.

Sample No	Age of the parents (Yrs)	Embryo obtained	Embryo survived	Age of the parents (Yrs)	Embryo obtained	Embryo survived
1	2-3	128	33	3-4	106	42
2	2-3	182	168	3-4	285	110
3	2-3	50	32	3-4	357	212
4	2-3	348	180	3-4	270	62
5	2-3	84	47	3-4	168	102
6	2-3	149	135	3-4	409	315
	Total	**941**	**595**		**1595**	**843**

Table 3: Embryos obtained from the Mut/Mut and WT/Mut parents aged between 2-3 and 3-4 years (Values are mean SE±. N=7 [MUT X MUT]), N=6 [WT X MUT] * ($P<0.05$)

Sr. No.	Mating crosses	2-3 years	3-4 years
1	Mutant X Mutant	102.71±14.74	105.28±22.33*
2	Wild type X Mutant	156.83±42.43	265.83±46.30*

Table-4: Embryos survived from the Mut/Mut and WT/Mut parents aged between 2-3 and 3-4 years (Values are mean SE±. N=7 [MUT X MUT]), N=6 [WT X MUT].

Sr. No.	Mating crosses	2-3 years	3-4 years
1	Mutant X Mutant	74.71±13.39	64.42±16.85
2	Wild type X Mutant	99.16±28.38	140.5±42.36

C, 2020). The old breeder fish shows reduced reproductive performance after two years (Castranova D and Wang C, 2020) and do not spawn when mate naturally. In such a situation, reviving the zebrafish line using natural mating becomes difficult. In the present study, 12 old non-productive zebrafish breeder lines were revived and propagated using the in-vitro fertilization technique. It is also noticed that the aged males produced good semen indicating their breeding potential in old age however, watery and degenerated egg clutches were obtained from the majority of the old females revealing a decline in the spawning performance. These observations were supported by the earlier reports (Castranova D and Wang C, 2020).

CONCLUSIONS

In vitro fertilization in zebrafish proved to be a very useful approach for propagating zebrafish lines from aged animals and/or animals with poor or non-productive performance. In the current study, twelve aged and non-productive zebrafish lines were effectively reproduced using in vitro fertilization.

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Ethical Approval

The necessary approval was obtained from the Institutional Animal Ethics Committee (IAEC), TIFR, Mumbai.

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