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Maintenance and Breeding of Zebrafish under Laboratory Conditions for Animal Research

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ABSTRACT

Background: Zebrafish is widely used in biomedical research to explore several diseases and abnormalities. A thorough understanding of husbandry is required to maintain effectively, breed and produce healthy and diversified colonies. Diet, age, size, light exposure, mating behaviour, tank temperature and time used for egg formation are all factors that influence the breeding process.

Methods: The fishes were kept in the zebrafish housing system with frequent water quality checks and a breeding duration of 7 days. Developing eggs and larvae were photographed and further processed with image view software. The normal development of zebrafish goes through seven stages: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching stage.

Result: The average number of eggs deposited per female in the experiment was 341±4.21, with 318±4.81 viable eggs. 298±4.99 eggs hatched after 72 hours and 270±4.34 fingerlings survival was recorded after a week. The present paper discusses the care of zebrafish, setting up and maintenance in laboratory conditions. Once the system is standardized, it is easy to access healthy eggs, larvae and adults throughout the year for research and teaching purposes.

Key words: Biomedical application, Breeding biology, Embryonic development, Viable embryos, Water quality parameters.

INTRODUCTION

The zebrafish, formerly known as *Brachydanio rerio*, is a tropical freshwater teleost belonging to the Cyprinidae family and Cypriniformes order. No animal model is completely perfect for exploring human diseases. Because of ethical concerns, the search for new animal models has increased worldwide. The zebrafish *Danio rerio*, which has a fully sequenced genome and human-like features, is an effective animal model for studying human diseases. The zebrafish exhibits many features of vertebrate models apart from physiological and anatomical characteristics of higher organisms that attracted scientists worldwide for its use in biomedical research (Zhao *et al*., 2015). It is very easy to handle and care for zebrafish because its short life cycle, small size, good fecundity and optical clarity of embryos make it suitable for toxicological, developmental and pathological research. Their transparency enables the monitoring of developmental processes using non-invasive imaging and tracking of protein/cell markers in the body system (Spitsbergen, 2007). Model zebrafish can be developed diabetes, lipid-related diseases (Longkumer *et al*., 2022) and cancer as in human beings. Its embryo is an excellent vertebrate system for studying the cellular and molecular functions of genes associated with Alzheimer's disease (Koehler *et al*., 2018). It has also increasingly been used as a host for studies on infectious diseases. The behavioural responses of zebrafish to heavy metals and stress and catfish have been reviewed (Sahoo *et al*., 2017; Huang *et al*., 2021). Many drug compounds can be screened primarily through toxicological studies in carps, saving money and time (Kumari and Chand, 2021). For toxicity testing of water, the zebrafish embryo was included in the ISO test (ISO 15088:2007). Understanding and producing healthy stock in laboratory conditions becomes vital in light of the above utility of zebrafish and its ever-expanding application. A laboratory environment resembling zebrafish's natural habitat is necessary for optimal stock production. It has been shown that the agro-climatic conditions for zebrafish development can be maintained by utilising the zebrafish housing system (Model-NT-ZB-11; Make-Narshi Technologies). The increase in demand for zebrafish is mainly satisfied by wild catch; such housing systems are helpful in ensuring the long-term viability and sustainability of ichthyofaunal resources. The paper then illustrates the basic process involved in the successful breeding of zebrafish.

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MATERIALS AND METHODS

Animals

Adult zebrafish of both sexes were procured in bulk from Aqua Fish and Pets, Jorhat, Assam, India. Then they were acclimatised and cultured in a zebrafish housing system (Model-NT-ZB-11; Make-Narshi Technologies). Polycarbonate

fish tanks of sizes 1 and 3 litres were maintained under a 14h/10h: day/night photoperiod cycle. Adult zebrafish were fed three times a day and randomly selected for the experiments. The experiment was conducted during 2019-2021 at the Department of Zoology, Nagaland University, India.

Reagents

All chemicals used were of reagent grade and obtained from Sigma-Aldrich. All solutions were prepared with deionized water.

Preparation of embryo medium

After fertilisation, the eggs were placed in an incubator (~28.5°C) for 72 hours, when the larvae hatched. The embryos were reared in an embryo medium prepared as per the cold Spring Harbor Protocol, 2011 (Williams and Renquist, 2016).

Water quality parameters

The water temperature and pH measurement were carried out with commercially available instruments ISTA LCD Digital aquarium thermometer and digital LCD pH meter pen (Indiefur enterprises, New Delhi). Salinity, conductivity, dissolved oxygen, water Hardness and alkalinity were measured weekly using the Aqua Merck compact Laboratory for water testing kit (Merch, KGaA, 64271, Darmstadt, Germany). Salinity levels, conductivity; total hardness ($CaCO_3$); total alkalinity was checked and maintained as per standard methods Avdesh *et al*. (2012); Longkumer *et al*. (2020).

Zebrafish housing system

The zebrafish housing (Model-NT-ZB-11; Make-Narshi Technologies) is well equipped and customised with a high-density rack and a semiautomatic recirculating water system with all essential, necessary water filtration and disinfection. Chemical, mechanical and biological filtrations, as well as UV radiation, are used in the water filtration system, which is then channelled into each individual fish chamber. The fishes (n=7) were kept in a 1-litre individual fish tank of zebrafish housing system while the temperature was maintained at 28±2°C, using a submersible heater (300W). Photoperiod was maintained with a multi-circuit digital switch. The pH of the housing system was kept at 7.3.

Feeding of zebrafish

Adult zebrafish were fed three times a day alternately with commercially available feeds procured from Perfect Companion Philippines Corporation. The feeding amount was given so that it was consumed within 10 minutes. After hatching out from their yolk sac, developing embryos were fed with egg yolk and later with brine shrimp and paramecium. Zooplanktons such as paramecium, daphnia and rotifer were cultured in the laboratory and provided to the developing larval zebrafish along with the commercially available diets (Longkumer *et al*., 2018).

Breeding conditions

Reproductively matured adult females and males were selected for breeding experiments. The breeding set-up included a soft brush, petri dish, breeding chamber, egg separator, pebbles and dropper (Fig 1b-1d). The zebrafish were kept in the individual breeding chamber late in the evening as they lay their eggs during the early dawn period with the initiation of white light. A plastic eggs separator that provides a medium of separation for the fish to access the eggs were utilised. The collected eggs were kept in a petri dish with methylene blue to prevent mould or fungal infections. The housing combination ratio used for males and females was 2:1 as the extra number of males increases fertilization efficiency, as recommended by Tsang *et al*. (2020).

Larval rearing

The zebrafish larvae were kept in round Petri dishes with a 50% change of the medium daily. Initially, until the 4^{th}-5^{th} days, the embryos feed off their yolks. The young larvae were fed dry food (~100 microns in size) and live foods such as paramecium and rotifers. The larvae were kept in the Petri dishes for 14 days, after which they were transferred to a 1-litre fish tank associated with the zebrafish housing system, where they were kept until further development. The food size was increased with the growth duration of the larvae, as recommended by Avdesh *et al*. (2012).

Image pre-processing and processing

An Olympus Stereo Zoom microscope (Model SZX10) was used for imaging and sorting out the eggs from other wastes and separating them from the damaged eggs. A labomed cxl binocular microscope having magnification (4X, 10X, 40X and 100X) attached with sony digital camera model E31SPM20000KPA (USB 2.0) and Image J software installed computer desktop (Intel Quad Core 2.8 GHz desktop computer with 64 GB RAM under Windows 7) was used for pre-processing and processing of the videos and images of the developing embryos and larvae at different hours of development (Fig 1e-1h).

Ethics statement

All animal experimental procedures were conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Ethics Committee (IEAC), Nagaland University, Lumami with the IAEC approval no. NU/ZOO/IAEC/Meeting No 1/2020, Protocol No. 04.

Statistical analysis

All statistical analysis was performed in GraphPad Prism 5 software (Trial version) as the mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Water quality parameters

The zebrafish housing system's water quality parameters showed optimal conditions for maintaining and breeding

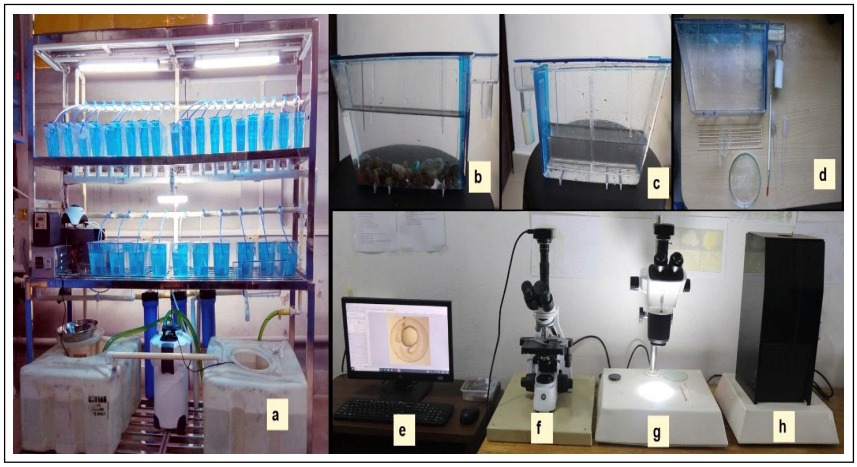
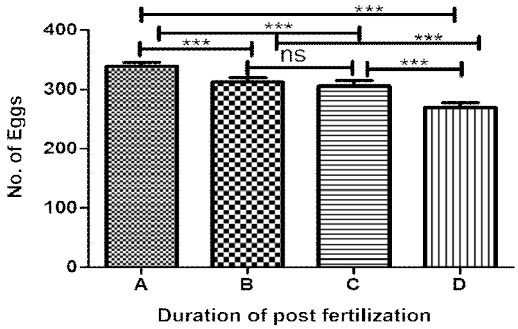


Fig 1: (a) Zebrafish housing systems; (b-d) Experimental set up for the zebrafish breeding chamber; (b) Pebbles as egg barrier; (c) Plastic egg separator; (d) Plastic egg separator, petri dish, brush and transfer pipette dropper; (e-h) Imaging System; (e) Desktop with Image J Software; (f) Cxl Binocular microscope; (g) Olympus stereo zoom microscope; (h) Dark chamber for adult fish imaging.

under laboratory conditions. The water temperature was 28°C (25-30°C), while the pH value was 7.3 (6.5-8.0). Nitrite, nitrate and ammonia were all measured to be 0 ppm. The total hardness and conductivity of the system water were in the range of 75-200 mg/l of $CaCO_3$ and 200-1500 µS/cm, respectively. Dissolved oxygen was kept at not less than five mg/l. The photoperiod was 14 hours of light and 10 hours of darkness.

Reproductive ability and stages of embryonic development

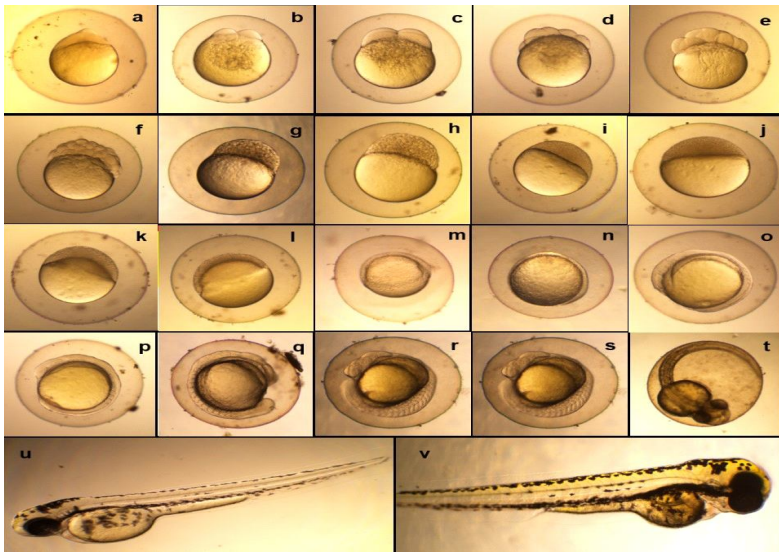
Experimental set-up with a male: female breeding ratio of 2:1 showed the eggs spawned from each breeding chamber to be 340 ± 4.89. Unfertilized eggs turned milky white, while the fertilized eggs were clear and transparent. Averages of the number of viable eggs, hatchability after 72 hrs and fingerings survival after one week were recorded (Fig 2). The embryonic development of zebrafish follows seven distinct stages in the sequence the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching (Fig 3). The zygote stage marks the successful fertilization and the beginning of embryonic development where the cytoplasm is found to accumulate at the animal pole and it involves partial cleavage resulting in the one and two-cell stages, occurring within 0-0.75 hours post-fertilization (hpf). Cleavage follows the zygote stage where 4 and 8 blastomeres were discovered at 1.25 and 1.50 h (hpf). Cleavage occurs on two parallel planes in the 8-cell stage, dividing the blastodisc into two 2×4 blastomere arrays. Two parallel planes on either side of the second cleavage plane generate a 4x4 array of cells in the 16-cell stage. Between the first and third cycles, the 32-cell stage cleaves along parallel planes rather than two, resulting in 4×8 blastomeres. Horizontal cleavage occurs in the 64-cell stage. Blastula stages involve the appearance of 256 blastomeres resulting from late cleavage and are recorded within 2.25-5.25 hpf. Oblong and sphere stages are also part of the blastula stages in which the yolk sac compresses the animal-vegetal axis of the developing fetus and in the later further shortening



A) Eggs laid per female (B) Viable eggs per female (C) Hatchability after 72 hours (D) Fingerlings after one week. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-hoc test (*p<0.01); ns: Not significant.

Fig 2: Reproductive ability of zebrafish (n=6).

along with the animal vegetal axis results in a smooth and nearly round late blastula. The gastrula stage is marked by the beginning of various degrees of epiboly. The blastoderm is shaped like an inverted cup with a constant thickness and a margin that is 30% of the distance between the animal and vegetal poles. Blastoderm is consistent in thickness in the next stage by 50% epiboly. The epiblast, hypoblast and evacuation zone occur on the dorsal side and the notochord rudiment finally separates from the segmental plate at around 90% epiboly. In the segmentation stage, the first somite furrow begins to appear. 18, 21 and 26 somite stages were detected at 18, 19.5 and 22 hpf, respectively. During the 21- somite stage, the posterior trunk begins to straighten. The Pharyngula stage follows the segmentation, which occurs within the 24-48 hpf; here, distinct pectoral fin development, increased embryo spontaneous movement and the detachment of tail from the yolk is recorded. The circulatory system is well marked, with the distinct heartbeat and the developing embryo exhibiting early pigmentation marks. The hatching stage completes the embryonic



(a) 1 cell stage; (b) 2 cell stage; (c) 4 cell stage; (d) 8 cell stage; (e) 16 cell stage; (f) 32 cell stage; (g) 64 cell stage; (h) 256 cell stage; (i) Oblong stage; (j) Sphere stage; (k) 30% epiboly stage; (l) 50% epiboly stage; (m) 75% epiboly stage; (n) 90% epiboly stage; (o) 3 somite stage; (p) 6 somite stage; (q) 18 somite stage; (r) 21 somite stage; (s) 26 somite stage; (t) embryo at 24 hpf; (u) embryo at 72 hpf; (v) larvae at 96 hpf.

Fig 3: Different stages of the embryonic development of zebrafish.

development, taking place within 48-72 hpf. The embryos twisted up and down inside the egg for a few hours after the egg membrane split before hatching and finally escaping out of the egg. After the embryo hatched, the yolk sac of the growing larva shrunk. The eyes became more distinct as they reabsorbed and the barbells surrounding the mouth grew more prominent and developed.

Water quality management is a complex and vital aspect of a recirculating system involving several components influenced by both chemical and physical elements in the environment. Temperature, pH, conductivity, total hardness, dissolved oxygen and nitrogenous waste are parameters that stand out. Temperature is a parameter that needs daily monitoring and plays a vital role in normal development, particularly spawning. Temperature directly affects the development rate and hatching success of fish eggs. An optimum level of temperature is required for each species for the highest hatching rate and proper development (Chacko and Sekharan, 2022). In zebrafish, the most favorable and accepted temperature for zebrafish culture is 28°C. The pH also significantly affects several biological processes in zebrafish, which may, in turn, alter their ability to breed. Several events, such as feeding leftover; waste excreted from the fishes followed by the oxidation of the nitrogenous wastes from the fishes by the nitrifying bacteria in the biofilter, alter the pH of the system leading to a decrease in the pH and as such the external interference in the form of addition of buffers becomes essential. When the pH drops below the optimum range, the risk is the subsequent ammonia spike in the system water, which is harmful to the health of the fish. Further, the pH alteration harms the fish and disturbs the microbial community that supports them (Kent and Varga, 2012). In terms of dissolved oxygen, zebrafish should be kept in water with a concentration of at least 6.0 mg/L (Longkumer *et al*., 2020). The imbalance results in the highest mortality in damage caused to the fish by the various water quality parameters (Sahoo *et al*., 2017). All freshwater fish invest energy to balance their internal salts and water and the saltiness of the water in which they live (Engeszer *et al*., 2007). Zebrafish systems monitor and regulate salts in fish water, primarily sodium chloride, to minimize the expense of osmoregulation for fish to maximize energy utilization for growth and reproduction. The spectrum and intensity of light produced by artificial lighting and the length of the light phase, *i.e*., the photoperiod employed, can significantly affect spawning, hatching of eggs, larval development and the growth rate of fry and the behavior of zebrafish. In line with such factors, the photoperiod in laboratory conditions is ideally set at 14 and 10 hours of light and darkness, respectively. It is advised that ammonia, nitrite and nitrate levels in the source water be kept at minimal levels. Ongoing monitoring should focus on unionized ammonia levels of <0.05 mg/L (or ppm), nitrite concentrations of <0.1 mg/L and nitrate concentrations of <50 mg/L.

Zebrafish housing systems constantly evolve with various new technological advancements, opening the doors for sophisticated life support systems. These systems have multiple steps of excellent filtration methods, consisting of a mechanical filter, an activated carbon filter, a biological filter and an ultraviolet (UV) sterilizing unit. The current system has a semi and fully automated water quality

monitoring system, automatic photoperiod management and temperature management, which can all be pre-set in the method depending upon the requirements of the fish and the experiment carried out. Further, specific and specialized spawning systems and automatic feeding have also been made available.

CONCLUSION

The aim of the various housing system is to provide a stable and favourable environment that enables the production and maintenance of healthy fish stock. Keeping this in mind, the zebrafish housing system was chosen and established, making an effort to make the maximum output for its aid as researchers and enabling a state-of-the-art zebrafish housing system. Information on early egg and larval ontogeny are of critical importance in understanding the basic biology, dietary requirement and environmental preference of a particular species. A clear understanding of the husbandry of zebrafish enables the best possible scientific research to be achieved in which it is used as a model organism. Zebrafish as a model organism finds its application in a diverse field of research studies ranging from developmental genetics, modelling of various human diseases, toxicological studies and drug discovery. The breeding of zebrafish involves several factors. Once it is standardised, one can successfully maintain and have a healthy number of zebrafish in various developmental stages that can be utilised for multiple research experiments.

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Conflict of interest

All authors declared that there is no conflict of interest.

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