Video Article

Regular Care and Maintenance of a Zebrafish (*Danio rerio*) Laboratory: An Introduction

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Abstract

This protocol describes regular care and maintenance of a zebrafish laboratory. Zebrafish are now gaining popularity in genetics, pharmacological and behavioural research. As a vertebrate, zebrafish share considerable genetic sequence similarity with humans and are being used as an animal model for various human disease conditions. The advantages of zebrafish in comparison to other common vertebrate models include high fecundity, low maintenance cost, transparent embryos, and rapid development. Due to the spur of interest in zebrafish research, the need to establish and maintain a productive zebrafish housing facility is also increasing. Although literature is available for the maintenance of a zebrafish laboratory, a concise video protocol is lacking. This video illustrates the protocol for regular housing, feeding, breeding and raising of zebrafish larvae. This process will help researchers to understand the natural behaviour and optimal conditions of zebrafish husbandry and hence troubleshoot experimental issues that originate from the fish husbandry conditions. This protocol will be of immense help to researchers planning to establish a zebrafish laboratory, and also to graduate students who are intending to use zebrafish as an animal model.

Video Link

The video component of this article can be found at https://www.jove.com/video/4196/

Protocol

1. System Maintenance

1. Zebrafish are kept in a circulating system that continuously filters and aerates the system water to maintain the water quality required for a healthy aquatic environment. The circulating system also helps to filter excess food and fish excreta. Different companies provide zebrafish systems but we use systems from Aquatic Habitats, USA in our laboratory. The room temperature or the tank temperature is generally maintained between 26-28.5 °C and the lighting conditions are 14:10 hr (light: dark). A zebrafish system from Aquatic Habitats (*e.g.*, Benchtop system) costs ~9,000 USD. This benchtop system with two shelves can hold six 10-liter, twelve 3-liter, or twenty 1.5-liter tanks on each shelf. Multiple lines of fish (*e.g.*, transgenic, mutant, wild type) can also be housed on the same system.
2. A set of different kinds of filters are used in the system. In our system, water from all the tanks passes through a 120-micron filter pad, 50-micron canister filter, biological filter , active carbon absorption filter and UV disinfection filter before being circulated back into the tank. De-chlorinated/aged water is used in the zebrafish system. Water can be de-chlorinated by ageing for at least 48 hr. Under ideal conditions, water should be kept in a reservoir with a pump circulating the water to keep it warm, and expedite the de-chlorination.
3. The pH of the system water should be checked daily and maintained between 6.8 and 7.5. When necessary, sodium bicarbonate should be used to increase the pH.
4. Fish tanks should be cleaned regularly. To clean a fish tank, close the water flow to this tank, drain excess water by tilting the tank backwards and remove the tank carefully from the system. Dirt and algae growth will be apparent on the bottom and sides of the tank.

5. Place the baffle into a clean tank, and fill with de-chlorinated water (a.k.a. System water). Carefully transfer the fish into this tank with a fish net. Close the lid and transfer the name tag of the tank. Carefully place the clean tank into the system and switch on the water supply.

6. To decontaminate the fish net, spray with 70% ethanol, rinse in water, and let it dry before re-using. Remove the baffle from the dirty tank and spray both parts with 70% ethanol. Rinse thoroughly with tap water and allow the tank and baffle to dry fully before re-using.

7. The circulating system filters have to be checked and changed regularly to ensure their proper function. These filters should be changed regularly to ensure proper and clean water supply to all the fish tanks.

8. The 120-micron filter pad is usually repositioned or replaced daily; reposition towards the water flow to use it completely before replacing with a new one.

9. The Canister filter should be changed weekly. To change the canister filter, remove the filter unit by twisting anticlockwise with a wrench or hands. Place a plastic sheet or towel below to prevent or absorb water spillage. Replace the canister filter with a new one and fit the filter unit back into the system, carefully tighten with hands and a wrench if necessary.

10. The carbon filter should be changed fortnightly (every two weeks). To change the carbon filter, remove the carbon filter unit carefully with a wrench. Discard the used activated carbon and replace it with new activated carbon. Re-fit the carbon holder and place it back into the filter unit. Fit the filter unit back into the system. Switch on the system and check that water is flowing to the filter. If necessary, flush some water through the pipes to clean dirt in the pipes before fitting the filter into the system.

11. The biological filter should be cleaned every six months. A biological filter is generally located between the canister and carbon filter in the circulating system. To change the biological filter, remove the filter unit from the system. Release the pressure from the filter by pressing the pressure release button. Unscrew the filter with a wrench. Generally two people are needed for this step. Remove the lid of the filter unit. Empty the contents of the filter into a sieved container to separate the siporax from the water. Note: Siporax is a fine pore bio-filter media that has the ability to perform both nitrification and de-nitrification.

12. If the siporax is very dirty replace it with new. Fill the filter unit with system water (de-chlorinated water), close the lid, return the filter unit to the system and switch on the water supply. Note: In an acclimated aquarium, the siporax will be home to several nitrifying bacteria. These microorganisms are critical for maintaining the nitrogen cycle within the aquarium, and removing the primary housing for the biological filter (the dirty siporax) could result in a serious ammonia spike, followed by a nitrite spike while the new biological filter (with new siporax) is re-establishing. Both of these intermediate states of the nitrogen cycle may be toxic to aquatic organisms, and may kill zebrafish if not responded to appropriately. Therefore, it is important to have a secondary biological filter elsewhere in the zebrafish housing system (*e.g.*, in the collection tank in Aquatic Habitats system) to allow rapid repopulation of these important microorganisms on the new siporax.

13. UV filters are used to control system biological contaminants (such as bacteria) and should be replaced every nine-ten months. It should be noted that UV filter disinfection dose rate is ~110 mJ/cm^2 at the beginning of the lamp life and the dose rate decreases over the course of time, hence it is necessary to replace the globe even when it appears to still be functional.

2. Feeding

1. Zebrafish can be fed with dry food (food size from 100 microns for larvae to 300/400 microns for adult fish) or live food (brine shrimps). Brine shrimp (*Artemia sp.*) eggs are available from local pet shops and can be hatched in the laboratory by following the simple steps described below.
    a. Dissolve red sea salt in aged water by placing a beaker with salt on a magnetic stirrer for better solubility. Alternatively, salt can be dissolved in water by aerating it with the aeration tube. Instant ocean salt can also be used as a hatching solution if red sea salt is not available. Brine shrimps can tolerate a wide range of salinity; however, we hatch our brine shrimp (10-15 g) in 30-35 g/L red sea salt water. Note: In case brine shrimp eggs are not readily available in the local pet stores then encapsulated brine shrimps can also be used after "decapping" them in batches before adding them to the shrimp hatchery. This might also avoid frequent trips to the pet store.
    b. Fill the brine shrimp hatcher with salt water and add the shrimp eggs at a concentration of 1.2 tablespoons/liter. Aerate the hatcher vigorously with an air pump and leave the brine shrimp eggs to hatch for ~ 48 hr.
    Note: It should be noted that un-enriched brine shrimp lose nutritional value every hour post-hatching. Therefore, it may be appropriate to either hatch over 24 hr or enrich over 48 hr in order to obtain the maximum nutritional value from the brine shrimp.
    c. Waste water from the hatching system is added to quarantine waste to be disinfected with bleach and disposed later on.

2. To collect the brine shrimps, remove the air pipe and allow the culture to settle for 4-5 min but not for more than 10 min. The hatched brine shrimps gather at the bottom of the Hatcher.
    a. Collect brine shrimps using the tap at the bottom of the hatcher. Discard the initial flow which consists of un-hatched brine shrimp eggs.
    b. After discarding the un-hatched brine shrimps, collect the hatched brine shrimps which will be used as zebrafish food.
    c. Separate the brine shrimps from the salty water, using a brine shrimp collection net (~350 micron nylon mesh). Rinse the brine shrimps from the net into a container using system water.

3. The collected brine shrimps are generally present in high concentration at the bottom of the container giving it a rather orange colour.

4. Feed the brine shrimps to the zebrafish using a pipette or a dropper/squeezy bottle. The amount of food dispensed depends on the population size of individual tanks. The commonly accepted ratio for zebrafish is to receive 4% of body weight in food per day. Zebrafish should never be overfed as this may increase the nitrate level in the water, possibly affecting their breeding, or viability, as some fish may die due to overeating.
    a. Upon injecting food into the water, hungry fish swim to catch the brine shrimps.

5. Dry feeding can be performed using an Aquatic Eco-System's/Aquatic Habitats simple spring-based fish food dispenser. Alternatively, dry feeding can also be performed using a simple spoon or by cutting a plastic dropper diagonally with scissors to give it an appearance of a small spoon.

3. Breeding

1. Zebrafish initiate breeding at the onset of light. Fertilized eggs can be obtained either through in-tank breeding or pairwise breeding. While in-tank breeding is more labour-efficient and is implemented for regular embryo collection in our laboratory, pairwise breeding is preferred when genes or mutations are to be screened from individual fish.
2. For in-tank breeding, assemble the in-tank breeder and drop slowly into the fish tank after the onset of light. Alternatively, in-tank breeding set-up can be left overnight in the fish tank.
3. Leave the in-tank breeder for around 15 min to allow the fish to mate before removing the breeder from the tank and collecting the eggs.
4. Pairwise breeding is usually set up late in the afternoon after feeding.
5. Assemble the breeding tank and fill it with aged system water.
6. Transfer one female and one male to opposite sides of the breeding tank. Females can be distinguished from males because of their bigger underbelly. Males can also be distinguished from females because they are more slender and darker in colour than females. Moreover, males have more yellow colouration in the anal fin compared to females (see **Figure 1**). When in doubt look for the ovipositor in female zebrafish (see **Figure 4**).
7. Remove the divider the next morning shortly after the onset of light. Allow mating to occur undisturbed for 20 min or until sufficient numbers of embryos are laid at the bottom of the tank.
8. After breeding, return the fish to their tanks. Collect the eggs using a strainer.
9. Wash the embryos thoroughly with system water.
10. Transfer the embryos to a Petri dish by rinsing the strainer with embryo medium; a.k.a. EM3 (NaCl, 13.7 mM; KCl, 0.54 mM; MgSO$_4$, 1.0 mM; CaCl$_2$, 1.3 mM; Na$_2$HPO$_4$, 0.025 mM; KH$_2$PO$_4$, 0.044 mM; NaHCO$_3$, 4.2 mM).
11. Embryos can be observed under a microscope. Fertilized eggs are then separated from the unfertilized eggs using a needle and a pipette (see **Figure 3**).

4. Raising of Larvae

1. Fertilized eggs are kept in an incubator (~28.5 °C) for 72 hr until the larvae are hatched.
2. Now the larvae are out of the chorion and swimming freely they are ready to transfer to a main fish tank. Larvae need to be fed from 5 days post-fertilization (dpf) and are kept in embryo medium (composition described in Part 3, 10) or system water. Larvae can be kept in round dishes with ~50% or more of water therein changed on a daily basis. The water change should include removing dead or diseased larvae and any other debris.
3. Transfer the larvae gently into a tank containing a small sized baffle (of around 300-400 microns). Dead and diseased larvae should be removed and a few milliliters of water should be added slowly on a daily basis.
4. After 14 days, larvae tanks can be shelved into the system, and supplied with a small stream of cycling water (1-2 drops per second). As the larvae grow, water flow can be increased. Different sizes of baffles can be used depending on the size of the larvae (*e.g.* baffle size 300-400, 500, 700-750, and 1000 microns) and a normal plastic baffle should be used for the adult fish.
5. It usually takes 3 months for the embryos to develop into sexually mature adult.

Representative Results

Zebrafish housing and maintenance is easier and cheaper than traditional rodent models. Several thousand zebrafish can be housed in a small laboratory. As a result of this protocol, researchers will be able to manage a zebrafish facility which will provide healthy conditions to the zebrafish. In addition, the following illustrations will help identify fertilized eggs, adult zebrafish, and their food. An illustration of a male zebrafish (**Figure 1A**) and female zebrafish (**Figure 1B & 1C**) is shown to help researchers distinguish between a male and a female zebrafish for breeding purpose. **Figure 2** depicts a microscopic view of; brine shrimps at 12X (**Figure 2A**), a single brine shrimp at 90X (**Figure 2B**), and an unfertilized brine shrimp egg at 90X (**Figure 2C**). This will help in understanding the difference between unfertilized and fertilized brine shrimps for proper feeding purposes. Fertilized and unfertilized eggs are shown in **Figure 3**. **Figure 3A** illustrates a microscopic view of fertilized and unfertilized embryos. Unfertilized embryos are generally opaque and/or with ruptured cell(s) inside the chorion (black arrow) whilst fertilized embryos appears intact and growing to the next cell division state (for detailed reading of different stages of zebrafish embryos see[1]). Higher magnification view of a fertilized and an unfertilized egg is shown in **Figure 3B and 3C** respectively. **Figure 4** illustrates a female zebrafish's ovipositor to help researchers distinguish between a male and a female zebrafish.

Several critical problems that might occur in a zebrafish laboratory include blockage of water supply to individual/all tanks in the housing system, poor water quality, and leakage in pipes or reservoir of the circulating system. In addition, problems in obtaining embryos from breeding could be another concern. Troubleshooting of these issues is discussed below.
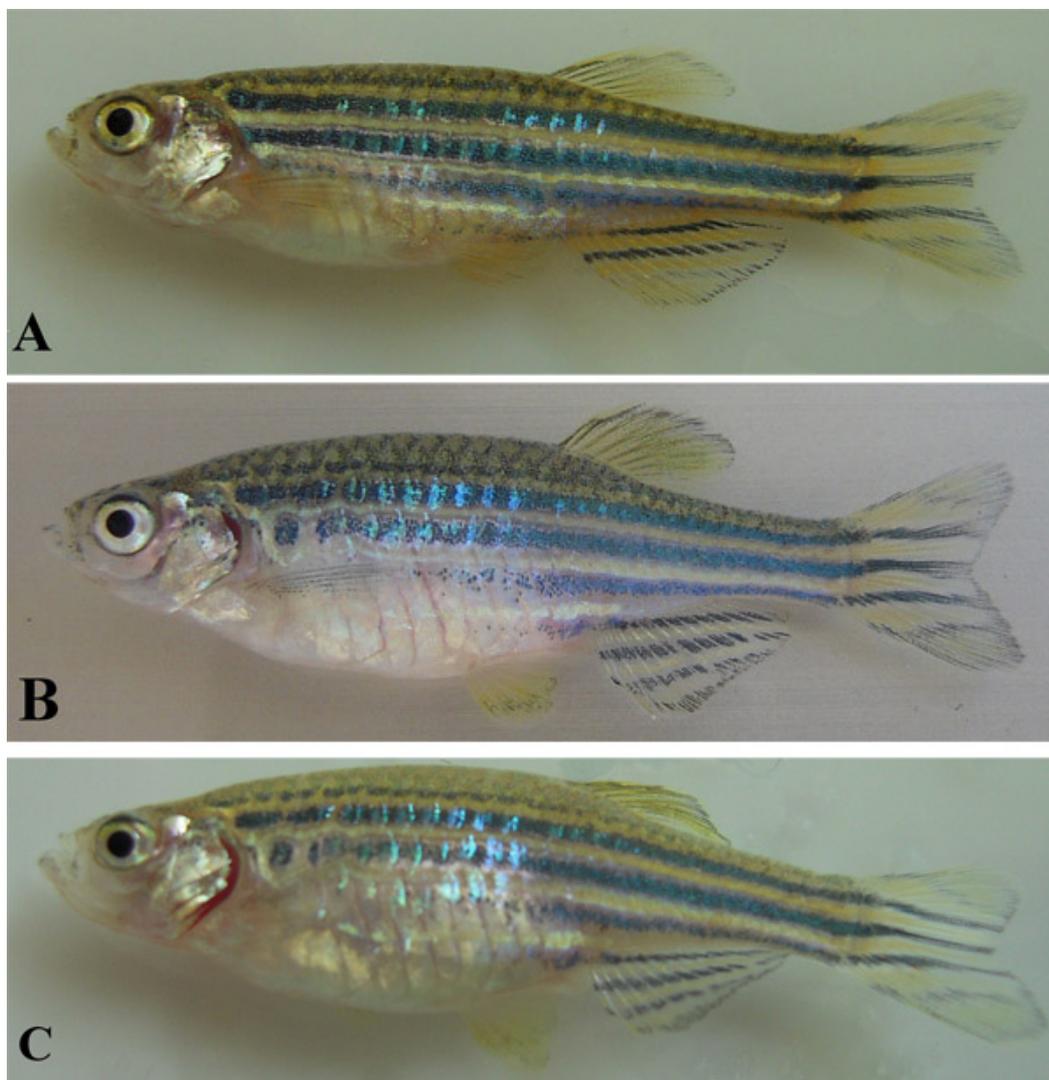
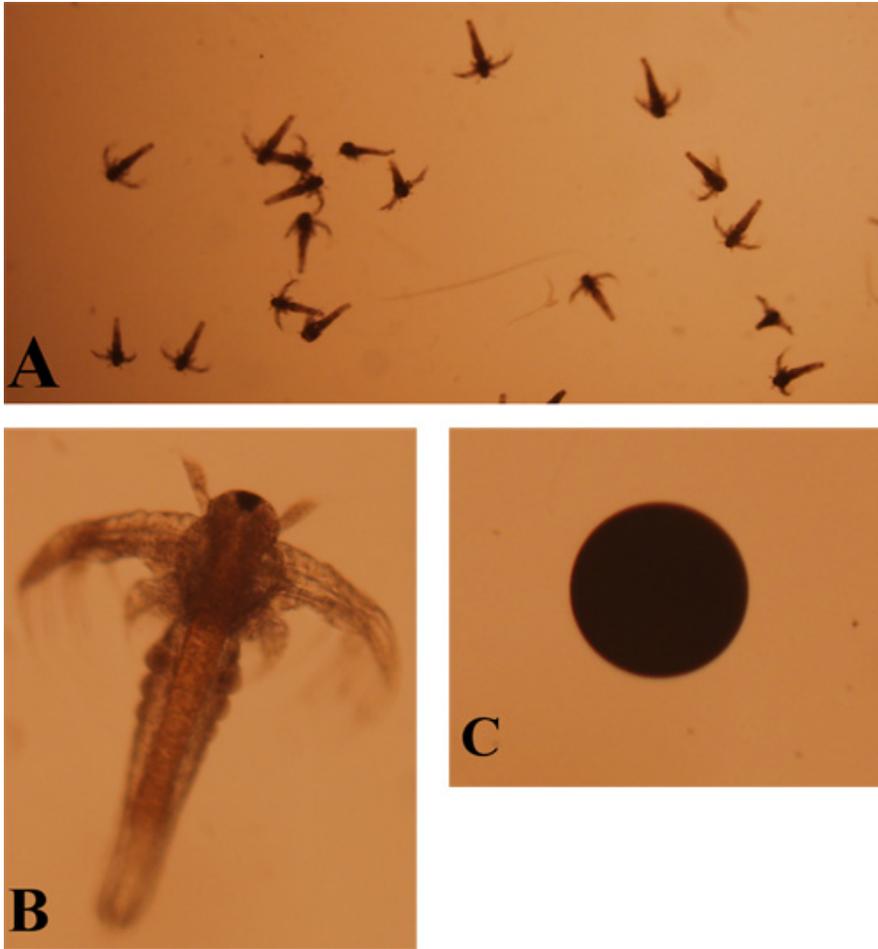**Figure 1.** An illustration of a male zebrafish (A) and female zebrafish (B, C).

Figure 2. A microscopic view of brine shrimps at 12X (A), a single brine shrimp at 90X (B), and an unfertilized brine shrimp egg at 90X (C).
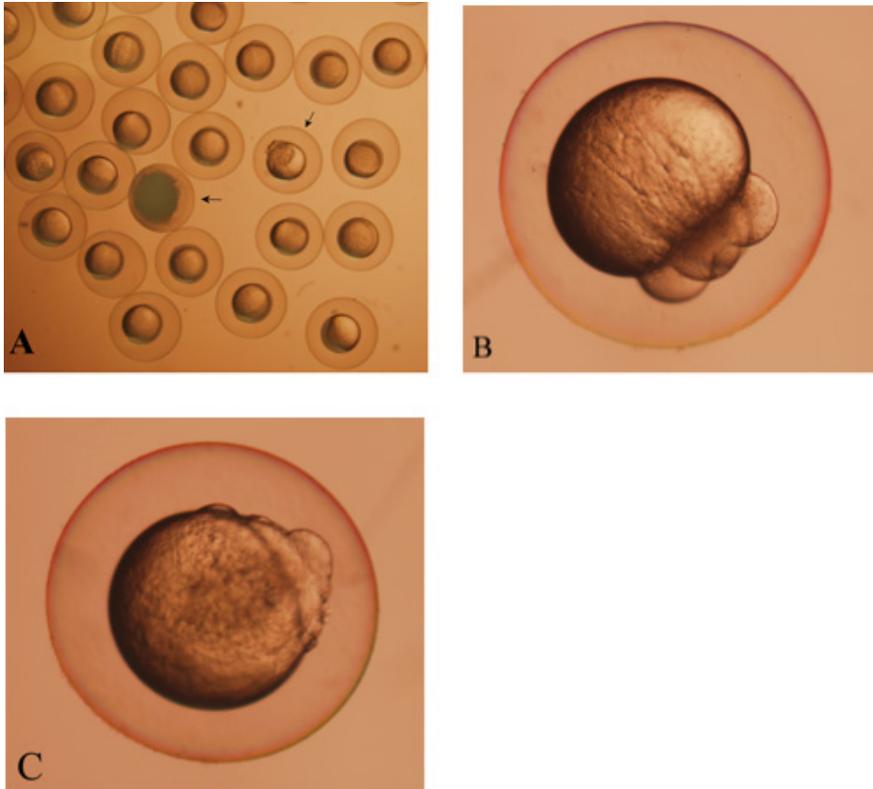
Figure 3. Microscopic view (16X) of fertilized and unfertilized eggs, where only two eggs are unfertilized, the unfertilized eggs are indicated with black arrows (A). Higher magnification view (90X) of a fertilized (B) and an unfertilized egg (C).

Figure 4. A female zebrafish's ovipositor (indicated with black arrow) illustration.

Parameter	Optimum range
Alkalinity	50-150 mg/L $CaCO_3$
pH	6.8-7.5 (6.0-8.5 tolerated)
Temperature	26-28.5 °C
Hardness	50-100 mg/L $CaCO_3$
Un-ionized Ammonia	<0.02 mg/L
Nitrate (NO^{3-})	<50 mg/L
Nitrite (NO^{2-})	<0.1 mg/L
Dissolved oxygen	>6.0 mg/L
Salinity	0.5-1 g/L
Conductivity	300 -1,500 µS

Table 2. Water quality parameters. Water quality parameters. Optimum range of environmental parameters in the zebrafish aquarium[11].

Discussion

Zebrafish originate from the Ganges river in northern India and are becoming popular in research in both their adult and larval stages[2], reviewed by Spence *et al.*[3]. Zebrafish possess several advantages over other animal models such as high fecundity, ease of maintenance, optical clearance of embryos, rapid embryonic development, and low maintenance cost. They are amenable to genetic manipulation[6] and suitable for high-throughput drug screening[4,5]. Their fertilization is external which is advantageous for their use by developmental biologists. Due to these favourable characteristics, zebrafish are gaining popularity in genetics[7], pharmacological[8], and behavioural research[9,10]. There are a number of challenges to maintaining a zebrafish facility and zebrafish husbandry to obtain embryos. Herein, we describe our experiences and recommendations in addressing these challenges and outline a protocol for system maintenance, feeding, breeding and raising of the larvae.

System maintenance

To maintain zebrafish in a healthy condition, it is important to provide them with a clean environment in a properly functioning aquarium system. An important part of this is changing system filters regularly so that all the tanks receive proper water flow and clean water. It is vital to avoid failure of the cycling water supply to each tank due to blocked system pipes. The pipes can be cleaned using a higher than normal water pressure and flow if blockage does occur. Ideally, around 10% of the system's water should be replaced daily to maintain good water quality. Alternatively, water can be replaced while changing the Canister or Carbon filter. This ensures that dirt deposited in the pipes connecting these filters is removed. The quality of water should be checked on a regular basis. Parameters such as alkalinity, pH, temperature, hardness, ammonia, dissolved oxygen, salinity, and conductivity should be considered as important factors in representing the quality of the system water (see **Table 2** for details). At the very least nitrate, pH, and temperature should be monitored on a regular basis to ensure good water quality for housing zebrafish. Ideal nitrate (NO_3^-) levels are <50 mg/L[11]; if high these levels can be reduced by replacing the water in the circulating system with the fresh system water. Occasionally, filters do not fit well and leak so it is recommended to check for any leaks after a filter change. If the water flow from the main reservoir is blocked either after changing the water pump or changing a filter, the water flow can be restored by either loosening or removing the filter for a few seconds to release any vacuum being generated in the pipes. The time required to change filters can vary depending on various factors such as total biological load on the system, cleanliness of other filters, and dirt deposited in the pipes. Hence, filters should immediately be changed if they appear dirty or if all the tanks are not receiving the correct water supply. It is also recommended that the fish net be cleaned with 70% ethanol, and rinsed in water to decontaminate it, and allowed to dry before being re-used. Drying ensures evaporation of ethanol which otherwise is toxic to fish.

Most of the zebrafish systems use de-chlorinated tap water; however, some systems use deionized water. It is important to keep the conductivity of the system water between 300 and ~1,500 µS as this reduces the energy the fish needs to maintain body salts. Therefore, zebrafish cannot be kept in deionised water unless salts are added to maintain the optimal conductivity levels. There is a risk of possible high copper concentrations in the system water if tap water is used because the carbon filter does not remove copper. Therefore, users should check for copper concentrations and avoid copper piping where possible.

Feeding

Zebrafish should never be overfed as this may increase the nitrate level in the water, possibly affecting their breeding[11], or viability, as some fish may die due to overeating. We recommend providing no more food during any one feeding than a tank of fish can finish within 10 min. It is very important to remove salt from the brine shrimps before feeding them to the zebrafish as excess salt concentration causes death. If more zebrafish eggs are required, fish can be fed three times a day. Cleaning the breeder fish tanks daily also improves levels of egg production.

When feeding on the Aquatic Habitats systems we usually turn off the water pump and air pump to allow the fish to eat the food for 10 min. This decreases the amount of food that is washed into the filters. However, users must be careful to remember to turn on these pumps again afterwards.

Breeding

Zebrafish are usually at optimal breeding condition between ~3 and 18 months of age. Pairwise breeding should not be performed for two consecutive days[11]; however, in-tank breeding can be performed daily as a tank can hold many fish which reduces the chance of the same pair of fish being bred for two days in a row. Breeding should be undertaken at regular intervals even if eggs are not required. This process will ensure the breeding cycle of the fish is maintained. It is recommended that there are more females than males in a breeding set-up. Male zebrafish change their female partners on a daily basis[12] which further supports this recommendation. Furthermore, within our laboratory we initially experienced problems with breeding, however, using more females than males in a breeding set-up helped solve the problem. Moreover, feeding with a high protein content diet and brine shrimp two-three times a day, mixing fish from different tanks (from different parents), maintaining the temperature of the breeding set-up between 27 and 28 °C, and squeezing the bellies of females with blocked ovary tubes using gentle massage further improved egg production. We recommend keeping a record of fish lines/ origins to avoid in-breeding between siblings. This further improves embryo production. Keeping a record of the number of embryos laid by fish from each tank is also recommended. This assists with keeping a track of the best breeding fish tanks and taking measures to improve breeding in the fish not laying eggs.

Raising of larvae

Feeding of larvae should commence from 5 dpf (days post fertilization). Young larvae can be fed with dry food of ~100 microns in size (*e.g.*, ZM100) or live food such as paramecium and rotifers (which stimulates growth). The food size can slowly be increased to 200 microns (*e.g.* ZM200) or 300/400 microns (*e.g.* ZM300). A population of adult fish should be around 6-7 fish per liter of water. This practice is recommended for better maintenance of BOD (Biological Oxygen Demand) to the tanks.

Disclosures

No conflicts of interest declared.

Acknowledgements

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