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Protocol

Husbandry of the African Turquoise Killifish *Nothobranchius furzeri*

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The African turquoise killifish (*Nothobranchius furzeri*) is an extremely short-lived vertebrate that has emerged as a powerful model organism for several research areas, including aging and embryonic diapause, which is the temporary suspension of embryonic development. The killifish research community is expanding and developing new solutions to improve the tractability of the killifish as a model system. Starting a killifish colony from scratch can present numerous challenges. In this protocol, we aim to highlight critical elements in building and maintaining a killifish colony. This protocol should help laboratories start a killifish colony and standardize aspects of killifish husbandry.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Brine shrimp eggs (Brine Shrimp Direct BSEP6LB)

Prepare brine shrimp as in Steps 1–13 of Protocol: ***Breeding and Reproduction of the African Turquoise Killifish*** **Nothobranchius furzeri** *(Chen et al. 2023). Specifically, add ~60 mL brine shrimp eggs and ~230 mL Instant Ocean salt to 14 L reverse osmosis H_2O in a 5-gal hatchery cone and aerate with bubblers (i.e., plastic tubing connected to an air pump) for ~48 h to allow the brine shrimp eggs to hatch. Attract the hatched brine shrimp for collection by shining a light at the base of the cone for 5–10 min. Use the stopcock valve at the base of the cone to release and collect the hatched brine shrimp. A bucket of hatched brine shrimp can be left on a countertop at fish room temperature (26°C–27°C), but it must be continually aerated with bubblers and used within 24 h. Filter brine shrimp before feeding by positioning an Artemia collecting net above a 4-L beaker. Pour the hatched brine shrimp into the filter and allow liquid to drain from the net. The hatched brine shrimp will remain in the net. Flip the net over into a clean 4-L beaker and use aquatic system water to rinse the brine from the net, resuspending the brine in new aquatic system water. This step prevents the buildup of ammonia in fry tanks. Pour hatched and recently filtered brine shrimp into a 500-mL squeeze wash bottle for feeding.*

Coconut fiber (Eco Earth Coconut Fiber, EE-8)

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From the African Turquoise Killifish collection, edited by Anne Brunet.

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Fill a beaker with commercially available coconut fiber. Add reverse-osmosis H_2O to the beaker until the fiber is completely wet. Autoclave the beaker containing coconut fiber for 1 h on a liquid cycle (~16 psi, 121°C), and then let the coconut fiber cool for ~24 h until cool enough to handle. Squeeze excess water out of the cooled coconut fiber and package into boxes, which can be stored on a countertop at room temperature for 3 mo. Once opened, a box of prepared coconut fiber should be used within 1–2 mo to prevent it from becoming too dry.

Dry fish pellets (Otohime fish diet, Reed Mariculture, Otohime C1)
Hatching solution for killifish <R>
Killifish (males and females for breeding)

Before beginning an experiment using killifish, it is critical to have a vertebrate animal protocol approved by the host institution to ensure their safe and ethical care and treatment.

Killifish embryo solution <R>
Sand (The Home Depot, Cemex 200000278)
Salt (Instant Ocean SS1-200)
Six-way test strip kits (Aquaneering STK6)
Sodium bicarbonate (Arm & Hammer CDC3320001961)

Equipment

Similar equipment can be substituted as desired.

Air pump (e.g., Hydrofarm AAPA25L)
Aquaneering filter bag (Aquaneering MFVB025C)
Aquaneering filter screens (Aquaneering 400-ZT080S400, 850-ZT080S850)
Aquatic system (e.g., Aquaneering)
Artemia collecting net (e.g., Amazon B09FL6L9WR)
Beaker (2-L, 4-L)
Bright overhead light
Central filtration recirculating system (e.g., Aquaneering)
Chemical weigh boats 85-mm long × 85-mm wide × 24-mm deep (Heathrow Scientific 120710)
Dissecting microscope (Leica M60)
Fish net (Pentair, Aquarium Net)
Glass Pasteur pipette (Fisherbrand 701865)
Hatchery cone (5-gal: e.g., Dynamic Aqua Supply CCH10)
Incubator with 17°C–40°C temperature range (e.g., Thermo Scientific Heratherm 50125590)
Lab squeeze bottle (BIPEE 500-mL Washbottle, Washbottle 500-1)
Labeling tape (ChromaLabel ACAL03800)
Petri dishes, 35-mm and 60-mm (Fisher Scientific 07-000-327 and 07-000-328, respectively)
Pipette pump pipettor with thumb wheel (Bel-Art Products F37898-0000)
Plastic Pasteur pipette (Globe Scientific 138090)
Plastic tubing (e.g., Advanced Technology Products B00E6BCV0G)
Sieve (OXO fine mesh strainer 38891)
Tanks, 0.8-L, 1.8-L, 2.8-L, and 6-L (Aquaneering ZT080, ZT180, ZT280, and ZT600, respectively)
Tweezers (e.g., Dumont #5 11251-30)

METHOD

Embryo Maintenance

After collecting embryos (see also Protocol: ***Breeding and Reproduction of the African Turquoise Killifish* Nothobranchius furzeri** *[Chen et al. 2023], maintain embryos in killifish embryo solution for 14 d (see steps below), and then transfer developed embryos to a dry substrate (see steps below).*

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Embryo Collection

1. Set up a breeding pair by combining at least one male and one female of similar size in a 2.8-L tank (Fig. 1). Alternatively, one male can breed with three to four females in a 6-L tank if larger clutches are desired. Both male and female breeders should be between 2 and 3 mo of age to obtain the maximal number of live embryos. Breed fish at the age when males are visibly sexually mature (i.e., when they show coloration). Although females do not show coloration, they become sexually mature at approximately the same age as males. Under the husbandry conditions described here, this generally occurs ∼1 mo after hatching. One breeding pair (one male and one female) can produce approximately 30 embryos per collection.

   Feeding breeding pairs more frequently than regular feeding generally results in improved fecundity.

2. Fill a single chemical weigh boat about halfway with dry, autoclaved sand (∼35 g; referred to as a "sand tray") and sink the sand tray to the bottom of the breeding tank (Fig. 1). For easy access, the sand tray can be placed at the front of the breeding tank. One sand tray is sufficient for a 2.8-L tank (single female) or a 6-L tank (multiple females).
3. After depositing the sand tray, wait 1 d to collect embryos. If desired, immediately replace the collected sand tray with a fresh sand tray for continuous daily breeding and embryo collection.
4. To isolate the embryos from the sand, first fill a 2-L beaker with aquatic system water. Place an appropriately sized sieve over the mouth of the bucket (Fig. 2). The liquid level in the beaker should be high enough that the basket of the sieve is mostly submerged. Gently pour out the contents of the sand tray into the sieve, and gently tap the sieve; sand should pass through the sieve while embryos remain trapped by the netting (Fig. 2B). Shine a bright light over the bucket to see the embryos. Use a Pasteur pipette to pick up and transfer embryos into a clean 35- or 60-mm Petri dish (use a 35-mm Petri dish for fewer than 30 embryos or a 60-mm Petri dish for up to 50 embryos).

   It can be difficult to see the embryos, but adjusting overhead lighting can improve visibility as the embryos will reflect the light (Fig. 2).

5. Under a dissecting microscope, select live embryos using a Pasteur pipette. Distinguish dead embryos by their lack of a chorion (Fig. 3A,B). Transfer live embryos to a clean 35- or 60-mm Petri dish of the appropriate size (see Step 4).
6. Remove the residual solution from the live embryos in the fresh plate (so the embryos are briefly left dry), and then immediately add fresh killifish embryo solution. Place clean embryos in an incubator at either 17°C–25°C (to facilitate diapause entry) or 26°C–27°C (to allow embryos to develop). The steps below are based on keeping the embryos at 26°C–27°C.

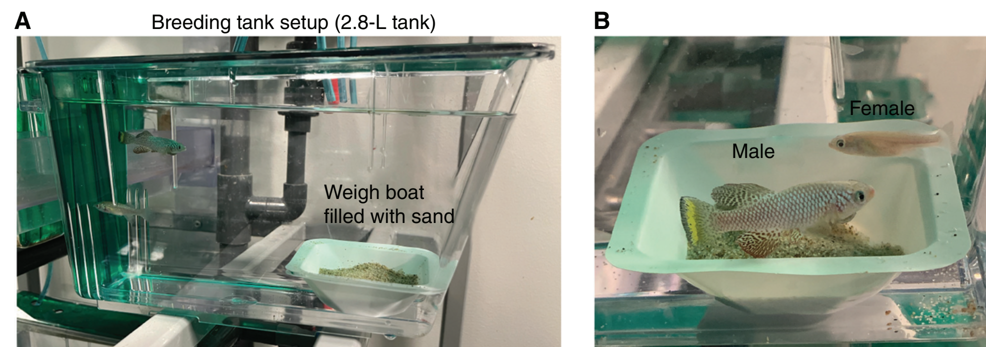

FIGURE 1. (*A*) Example killifish breeding tank (2.8-L tank) containing a sand tray. (*B*) Male and female killifish in the sand tray.

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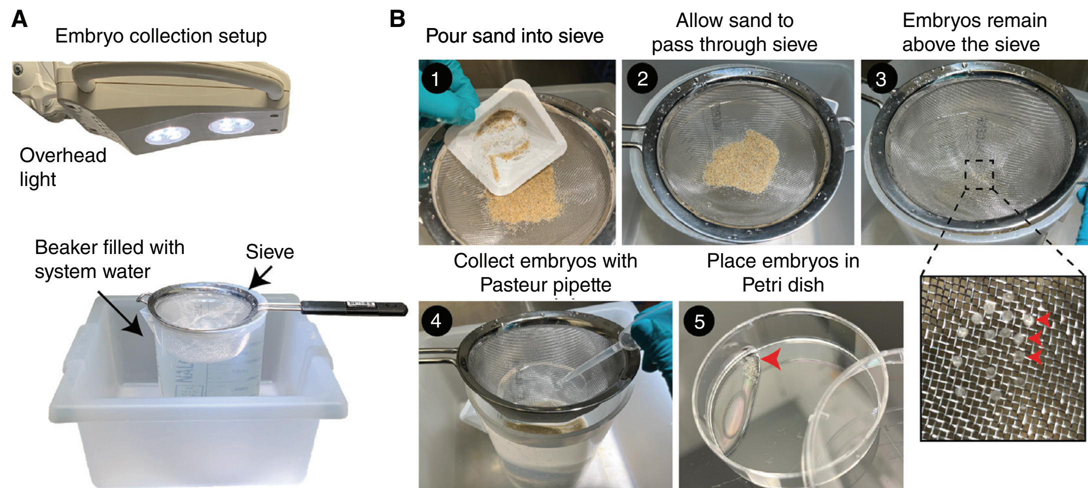

FIGURE 2. (*A*) Example setup for embryo collection, including crucial overhead lighting. (*B*) Example images of embryo collection from sand using sieve. Note that the embryos can be difficult to see, so adjusting overhead lighting such that it is optimal for your setup is necessary. Red arrowheads point to embryos.

Embryo Maintenance in Solution (14 d to >2 mo)

The temperature of the incubator influences the proportion of embryos that enter diapause. Incubators kept at 26°C–27°C facilitate a developmental trajectory whereby most embryos skip diapause and develop fully, whereas incubators kept at 17°C–25°C facilitate diapause entry (Hu et al. 2020; Astre et al. 2022). As a rule of thumb, to hatch embryos 1 mo from collection, maintain them in incubators kept at 27°C. If it is desired to hatch embryos at later time points (>2 mo from collection), incubate embryos at 25°C, and then 3 wk before the desired hatching time, transfer embryos to an incubator set to 27°C.

7. For each of the first 14 d after collection, remove dead embryos from the Petri dish (under a dissecting microscope) and exchange embryo solution using a Pasteur pipette. Embryos with visible contamination (i.e., fungus or parasites) should be removed even if they have a chorion. If more than a third of the embryos in the Petri dish are dead or show parasites, move the remaining live embryos to a new, clean Petri dish and exchange the embryo solution using a Pasteur pipette.

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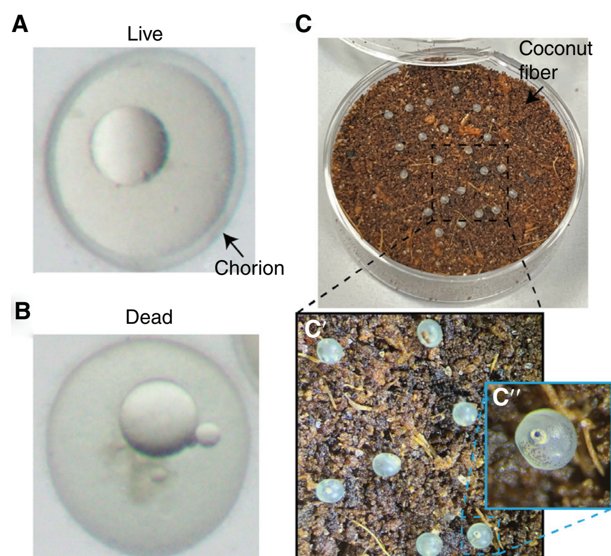

FIGURE 3. Examples of (*A*) live and (*B*) dead killifish embryos in embryo solution. (*C*) Developed embryos on coconut fiber. (*C*′) Embryos are spaced out. (*C*″) Embryos on coconut fiber are fully developed with clear eyes.

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The highest rate of embryo death occurs during the first 3 d after collection, so during this time it is important to place healthy embryos into a new Petri dish, remove dead embryos, and exchange embryo solution daily. After 3 d, removal of dead embryos and embryo solution exchange can be done every other day. Embryos must be kept in embryo solution for at least 14 d (at 26°C–27°C) to develop before being moved to the dry coconut fiber medium. Developed embryos can be stored in embryo solution for up to ∼1 mo. Embryos that are in diapause, developmental arrest, or otherwise undeveloped should be maintained in embryo solution until they develop (this can be much longer than 1 mo).

Embryo Maintenance on Coconut Fiber (14 d)

Embryos are maintained for 14 d on a dry substrate before hatching.

8. At the earliest, on the 14th day after collection, place developed embryos on coconut fiber or an equivalent dry substrate in a 35-mm Petri dish (Fig. 3C) as follows:

   i. Fill a clean Petri dish about halfway with an even layer of moist coconut fiber and press down the coconut fiber to make a flat surface.

   ii. Transfer developed embryos (which can be distinguished by their large black eyes) to the coconut fiber surface using a Pasteur pipette.

   Embryos that are in diapause, developmental arrest, or otherwise undeveloped should continue to be maintained in embryo solution until the development of large black eyes.

   iii. Ensure that the embryos are approximately evenly dispersed on the coconut fiber. Once the embryos have been placed on the coconut fiber, "water" the coconut fiber with embryo solution to keep it moist (use the laboratory squeeze bottle to add 1–3 mL of solution). Place the Petri dish with coconut fiber and embryos in an incubator at 26°C–27°C.

9. Check embryos on coconut fiber every other day to ensure the coconut fiber substrate stays moist. When the coconut fiber starts to dry out, add embryo solution to the edges of the Petri dish. "Watering" is typically required approximately every 4 d, depending on the humidity of the incubator.

10. Keep embryos on coconut fiber for at least 14 d before hatching. However, embryos can be stored on coconut fiber for ∼3–6 mo if "watered" around every 4 d. We observe a drop in hatching efficiency if stored for long periods (∼3–6 mo) on coconut fiber.

11. Allow the coconut fiber to dry (i.e., don't "water" it) for 5 d before hatching.

    We have observed the best hatching efficiency with embryos on dry coconut fiber (as opposed to embryo solution saturated coconut fiber). However, some embryos should still hatch if "watered" up to the hatching date.

Embryo Hatching and Fish Maintenance

12. At the earliest, on the 28th day after collection, use tweezers to gently pick up embryos from the coconut fiber–containing Petri dish and deposit them in a 60-mm Petri dish filled with 10 mL hatching solution for killifish. Dry embryos tend to float on the water surface unless actively pushed under the liquid. To favor successful hatching, completely submerge embryos in the hatching solution (Fig. 4).

13. Leave the Petri dish for hatching on a countertop, at room temperature, with the lid slightly open. Embryos should hatch within 24 h. We have observed variability in hatching efficiency that may be driven by environmental factors such as lighting, time of day, and temperature. However, we have not extensively tested the effect of these variables on hatching efficiency. After a 24-h incubation in hatching solution, most embryos should hatch.

14. Using a Pasteur pipette, transfer newly hatched fry to 0.8-L tanks containing a baffle, a 400-µm filter screen, and an 850-µm filter screen, and place the tanks in a central filtration water recirculating system. Apply a slowly dripping water flow (∼15 mL/min) for the first 2 wk after hatching, to avoid washing out fish from the tank. After 2 wk, remove the 400-µm filter and

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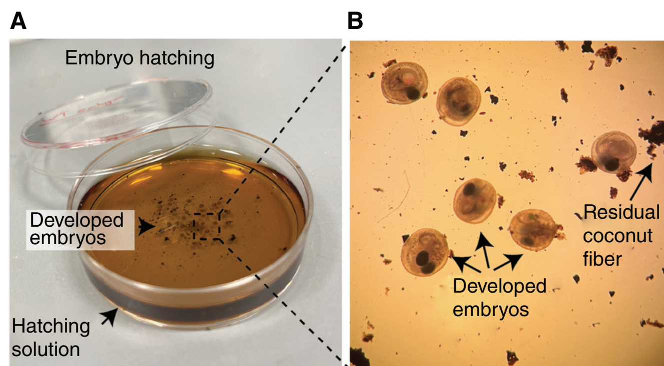

FIGURE 4. (*A*) Example image of embryos hatching in a 60-mm Petri dish with hatching solution. Developed embryos are placed in hatching solution. (*B*) Examples of developed killifish embryos submerged in hatching solution. When embryos are transferred from coconut fiber into hatching solution, it is normal for some coconut fiber to be brought into the hatching solution.

increase the flow rate to a slow flow (∼110 mL/min). Feed newly hatched fry two squirts of fresh brine shrimp (∼5–20 mL) daily until the fish are 4 wk old. Up to 10 newly hatched fry can be maintained in a single 0.8-L tank. At 2 wk after hatching, split to two fry per 0.8-L tank.

15. At 4–5 wk of age, transfer fish to 1.8- or 2.8-L tanks with a low-pressure steady stream of system water. Two large adult fish can be maintained per 2.8-L tank, although dominant male fish in this setup can be aggressive and can kill females or smaller males. A single adult fish can be maintained in a 1.8- or 2.8-L tank, and this is the preferred option for maintaining adult males.
16. For general maintenance, feed fish twice a day on weekdays and once a day on weekends (Žák et al. 2020). With each feeding, feed adult fish (>1 mo of age) a pinch (20–30 mg) of dry fish food (Otohime fish diet) and feed young fish (<1 mo of age) 5–20 mL of freshly hatched brine shrimp.

    The number of feedings, spacing between feedings, and amount per feeding can be changed as desired. However, these changes will likely impact growth, fertility, and life span, and it is important to record the conditions carefully.

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Maintaining the Fish Colony

General System Parameters and Maintenance

17. After hatching, house killifish at 26°C–27°C on a 12:12 h light-dark cycle in a central filtration recirculating system at a conductivity between 650 and 4000 µS/cm and a pH between 6 and 7.5, with a daily exchange of 10% of the water volume with reverse-osmosis H_2O.
18. Assess tank parameters daily. Note that killifish are healthy in system water with a wide range of conductivity (650–4000 µS/cm) (Dodzian et al. 2018; Astre et al. 2022). Higher conductivity can inhibit velvet disease (caused by parasites) (Astre et al. 2022). Note that the water flow rate into individual tanks is critical. A rapid flow rate can stress the fish and can wash young fry out of their tanks. High water flow also rapidly washes out food (i.e., brine shrimp) from the tank. However, too low a flow rate results in poor water quality within a tank. Having a low-pressure steady stream is ideal for most purposes. Also note that killifish are excellent jumpers and can escape into the sump system. To avoid fish escape, ensure lids are securely on tanks and monitor the water level in the tanks.
19. To ensure the health of the fish colony, perform polymerase chain reaction (PCR)-based diagnostic testing of both fish and water for potential pathogens (e.g., mycobacteria such as *Mycobacterium abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*, *M. marinum*, and *M. peregrinum* (Whipps et al. 2012); parasites; and viruses) on a quarterly basis.

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Water Quality

20. Exchange system water with fresh reverse-osmosis H_2O to the system daily. Add commercial marine salt and sodium bicarbonate as needed to maintain proper salinity and pH (see above). Filter water through mechanical filters (to eliminate large waste particles) and a biofilter containing bacteria that convert ammonia to nitrites and nitrates.
21. After biofiltration, treat water with UV sterilization to avoid contaminating organisms. To monitor water quality, measure pH and conductivity daily, and measure the levels of ammonia, nitrites, and nitrates in the system every week using six-way test strip kits. Large pH and conductivity swings as well as buildup of ammonia, nitrites, and nitrates are toxic to killifish and should be avoided by weekly system monitoring.

DISCUSSION

The African turquoise killifish (*N. furzeri*) is an extremely short-lived vertebrate that has emerged as a powerful model organism for several research areas, including aging and longevity. An *N. furzeri* line originating from a sample collected in 1970 in the Gonarezhou (GRZ) National Park, Zimbabwe is the predominant line used in experiments. This GRZ line is highly inbred, and therefore experiments with this line are done in a near-isogenic population of animals. There are many challenges in working with the killifish (Reichard et al. 2022). However, as the community of killifish researchers expands, new solutions to improve the tractability of the killifish as a model system have emerged. Previous reviews have provided in-depth information on husbandry (Polačik et al. 2016; Dodzian et al. 2018; Astre et al. 2022; Reichard et al. 2022). In this protocol, we have highlighted critical elements in building a killifish colony. Before building a killifish colony, it is critical to have an animal holding and breeding license approved by the host institution to ensure the safe and ethical treatment of laboratory animals.

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RECIPES

Hatching Solution for Killifish

Dissolve 1 g of humic acid (Sigma-Aldrich 53680) in 1 L of reverse-osmosis-treated H_2O. Autoclave at 15 psi for 20 min at 121°C. Store the solution at 4°C until use, as hatching efficiency drops when the hatching solution is equilibrated to room temperature before use. The solution can be used for at least 2 mo.

Killifish Embryo Solution

Dissolve two Ringer's tablets (Millipore 96724) in 1 L of MilliQ-purified H_2O or reverse-osmosis-treated H_2O. Sterilize the solution with 0.22-µm filters or autoclave the solution (15 psi, 20 min, 121°C). Dilute methylene blue (e.g., Kordon 37344) in the Ringer's solution at a final concentration of 0.002%–0.01% (e.g., 1 L of Ringer's solution with 100 µL of methylene blue). Protect the solution from light, and store at room temperature (good for at least 2 mo).

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