Protocol

Breeding and Reproduction of the African Turquoise Killifish *Nothobranchius furzeri*

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The successful breeding and reproduction of the African turquoise killifish *Nothobranchius furzeri* in a controlled laboratory setting are required to establish this fish species as a model system for studying vertebrate development and aging. Here, we describe a protocol to care for and hatch African turquoise killifish embryos, raise the juvenile fish to adulthood, and breed this species using sand as the breeding bedding. We also provide suggestions for generating a large quantity of good-quality embryos.

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 material 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

Blood worms (optional, see Step 21)

Brine shrimp eggs (technical grade; Brine Shrimp Direct)

Store for up to 3–6 mo at 4°C, as recommended by the manufacturer.

Coconut fiber

Add ∼4 L of coconut fiber (e.g., Zoo Med Eco Earth loose coconut fiber substrate for reptiles; Amazon B01CN8ZRYA) to a 4-L plastic container (Thermo Fisher 1201-4000). Fill the container with ∼2.5 L of reverse-osmosis-treated H_2O to soak the coconut fiber. The coconut fiber should have a thick mud-like consistency and not be overwatered. Cover the top of the bucket with a piece of aluminum foil. Do not tightly seal the bucket with foil; ventilation should be allowed. Autoclave at 15 psi for 30 min at 121°C. Place the autoclaved container at room temperature and leave the coconut fiber to cool down overnight (∼16–24 h). Clean empty pipette boxes with 70% ethanol and dry them with paper towels. When the coconut fiber is cooled, get a handful of the coconut fiber and squeeze out as much H_2O as possible. Add the coconut fiber to a clean pipette box. Close the box's lid, and store for up to 1 mo at 4°C or room temperature.

Dry feed pellets (Otohime C1, 2-kg bag, 580- to 840-µm pellets; Reed Mariculture)

Sieve the pellets through a 0.45-mm stainless-steel laboratory sieve to remove pellet powder. Keep the pellets that are >0.45 mm. This step helps reduce bacterial and fungal growth at the H_2O surface of the tank. Store the pellets for up to 2 yr at 4°C.

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

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Ethanol (70%)
Hatching solution for killifish <R>
Killifish (*Nothobranchius furzeri*) eggs

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>
Mild iodine solution <R>, freshly prepared (optional; see Step 50)
Ocean sea salt (Instant Ocean SS1-200)
Oxygen powder (optional; see Step 15)

Crush an oxygen tablet (e.g., O-tabs International oxygen tablet) to make powder.

Reverse-osmosis-treated H_2O
Sand (Cemex 30 Mesh Sand 200000278)

Filter the sand through a sand sieve (1-mm sieve size; e.g., OXO 38891) in system H_2O (using the setup described in Step 34). Remove the coarse grains trapped in the sieve and keep the sand that passes through the sieve. Autoclave the filtered sand at 15 psi for at least 20 min at 121°C before use. The sand can be autoclaved again and reused.

Equipment

Air pump for brine shrimp hatcher (Hydrofarm AAPA25L Active Aqua Air Pump, eight Outlets, 12-W, 25-L/min, 25-L; Amazon B002JPD76I)
Artemia collecting net (e.g., Amazon B09FL6L9WR; see Step 8)
Conical hatching jar (Pentair CCH10) with rigid clear tubing, 3/16″-OD × 3′-length (Pentair 16005)
Conical tube (50-mL; e.g., Fisher Scientific 14-432-22)
Curved, medium-point general purpose forceps (Fisher Scientific 16-100-110)
Flat, broad-tip forceps (Fisher Scientific 16-100-114)
Glass pipette (Fisher Scientific 13-678-30)
Incubator (e.g., Thermo Scientific Heratherm IMC18)
Lamp (e.g., Amscope LED-6W)
Microcentrifuge tube (optional, see Step 15; 1.5-mL)
Petri dishes (small, 35-mm × 10-mm, Thomas Scientific 627102; and large, 60-mm × 15-mm, Thomas Scientific 628102)
Pipette tip box (optional; see Step 15)
Plastic containers (4-L; e.g., Thermo Fisher 1201-4000)
Plastic transfer pipette (Globe Scientific 138090-250)
Plastic tub (e.g., Sterilite 1642)
Saran wrap or a transparent lid (see Step 16)
Soft fish net (e.g., the fish net in the Hygger carbon fiber six-in-one aquarium cleaning tool kit; Amazon B07MB183VL)
Stainless steel laboratory sieve (0.45-mm; e.g., Adamas-Beta 10-cm × 4.5-cm 40 Mesh 304 Stainless Lab Sieves; Amazon B07TMY1C1N)
Standard circulating H_2O system
Stereomicroscope (with bottom illumination; e.g., Olympus SZ61)
Tank filters (400-µm and 850-µm; e.g., Aquaneering ZT080S400 and ZT080S850)
Tanks (0.8-L and 2.8-L or 6- to 10-L; e.g., Aquaneering ZT080, ZT180, ZT280, ZT600, or ZT950)
Weigh boat (89-mm × 89-mm × 25.4-mm; Fisher Scientific 08-732-113)
Wide-opening baby brine shrimp net (e.g., SunGrow B01N2UBR67 and Aquaneering 2″ Net-SHFNT2)

METHOD

Fish (both juvenile and adults) are housed in a standard circulating H_2O system. Here, we refer the readers to Protocol: ***Husbandry of the African Turquoise Killifish (Nothobranchius furzeri)*** *(Nath et al. 2023) and other references (Polačik et al. 2016; Dodzian et al. 2018) for protocols on setting up the H_2O system. Typically, we maintain the H_2O system at 26°C, a pH of 6.6–7.5, and a daily 10% of H_2O exchange with H_2O treated by reverse osmosis. All animals are kept on a 12-h:12-h day/night cycle, with a light intensity of 10–60 lux.*

Perform all autoclaving steps at 15 psi for 20–30 min at 121°C. Perform all procedures at room temperature unless noted otherwise.

In the literature, there are many variations of how the African turquoise killifish N. furzeri *(hereafter, killifish) are hatched and raised. Here, we describe our typical method, but other methods also work well (Harel et al. 2016; Polačik et al. 2016; Dodzian et al. 2018; Žák et al. 2020).*

Brine Shrimp Preparation

1. Fill a conical hatching jar with 10 L of reverse-osmosis-treated H_2O.
2. Place three rigid, long tubes into the hatching jar. Ensure that the tubes are securely connected to the air pump with appropriate hosing and have unrestricted airflow.

   One of the rigid tubes should extend through the bottom of the hatching jar and be positioned directly above the exit valve. This setup prevents salt and brine shrimp eggs from packing above the exit valve, which results in poor hatch rates.
3. Add ∼350 mL of ocean sea salt to the hatching jar. Allow 5–10 min for the salt to dissolve.
4. Add 50 mL of dry brine shrimp eggs.
5. Hatch the brine shrimp for 48 h at ∼25°C.
6. Remove all the rigid tubes from the hatching jar. Allow the solution to settle for 10 min.

   The brown-colored shells of the hatched eggs will float to the surface, while most of the hatched, orange-colored brine shrimp will accumulate at the bottom of the cone.
7. For collection, position a 4-L plastic container underneath the exit valve and collect ∼1 L of the brine solution.
8. Position an Artemia collecting net above another 4-L plastic container.
9. Pour 0.5 L of the brine shrimp solution into the net. Allow the H_2O to drain from the net until H_2O is lightly trickling from the bottom of the net.
10. Pour clean system H_2O into the net until the net is full. Allow most of the H_2O to drain again.

    This step aims to rinse the brine shrimp in the net.
11. Flip the net over into a clean 4-L plastic container to release the shrimp. Use system H_2O to rinse out any brine shrimp that might be still on the net.
12. Repeat Steps 9–11 for the remaining 0.5 L of brine shrimp solution.
13. Add system H_2O so that the final volume of the prepared brine shrimp solution is 4 L, and place two rigid tubes (connected to the air pump) in the container for strong aeration. These brine shrimp are ready for feeding in a squeeze bottle or a plastic pipette at this density (∼800-shrimp/mL).

    Steps 8–12 aim to replace the brine shrimp hatching H_2O, which has high ammonia levels, with clean system H_2O. The wash step helps prevent ammonia buildup in the fish tanks after brine shrimp feeding. Because ammonia can still accumulate after preparation, brine shrimp feeding should occur within 30 min after the wash, and the brine shrimp should be rinsed again for subsequent feedings that occur 30 min after the initial wash. To rinse again, take note of the volume of the remaining solution, and repeat Steps 8–11. Fill the 4-L container with fresh system H_2O to the noted volume to keep the concentration of brine shrimp similar.

Fish Hatching

Healthy fish are essential for embryo production. Hatching fish at an optimal time is a key step in producing healthy and fecund fish. Premature hatching of embryos can prevent the fish from successfully inflating their swim bladders, leading to animals known as "belly sliders" that fail to swim properly.

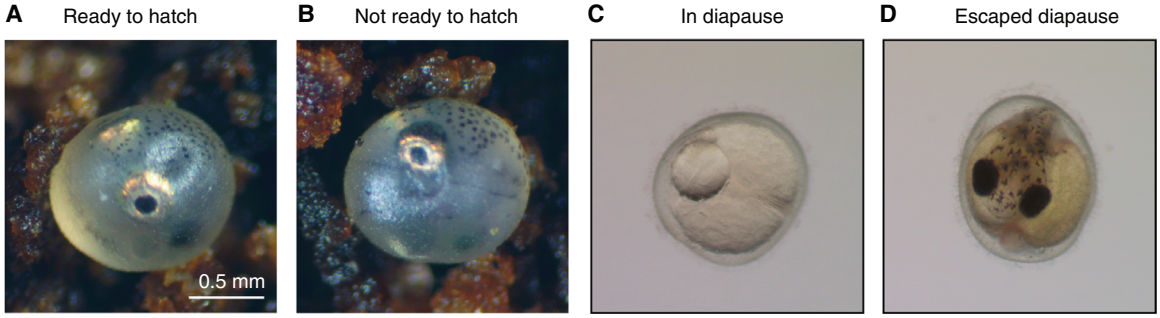


FIGURE 1. *Nothobranchius furzeri* embryos at different developmental stages. (*A*) An embryo with golden irises that is ready to hatch. (*B*) An embryo that is not ready to hatch, as its irises have tiny black dots within the golden region. (*C*) An embryo that has entered diapause. (*D*) An embryo that has escaped diapause at 14 d postfertilization.

14. Before hatching fish, ensure that embryos are ready to hatch by viewing them under a stereomicroscope.

    Using a lamp to provide illumination from above can enhance visualization of the fish eyes.

    The ready-to-hatch embryos should have eyes that have fully developed golden irises (Fig. 1A). When one looks straight down at the fish eyes under a stereomicroscope, one should not see a thick, black outline surrounding the golden region or any tiny black pigment spots within the golden region (Fig. 1B). The presence of a thick, black outline indicates that the embryos still need 4–7 d to further develop on a dry substrate (e.g., moist coconut fiber). Once the embryos are ready to hatch, proceed to Step 15.

15. Use a pair of curved forceps to carefully transfer the ready-to-hatch embryos into a Petri dish (see more details below) that contains hatching solution. As these embryos often float on the surface of the solution, gently rock the dish or use the curved forceps to make the embryos sink to the bottom of the dish.
    - For hatching one to five embryos, add 5 mL of cold hatching solution (optional: also add ~30 µL of oxygen powder; estimate the volume of the powder using a 1.5-mL microcentrifuge tube) to a 35-mm × 10-mm Petri dish.
    - For hatching five to 20 embryos, add 10 mL of cold hatching solution (optional: also add ~50 µL of oxygen powder) to a 60-mm × 15-mm Petri dish.
    - For hatching 20 to 50 embryos, add ~1 cm deep of cold hatching solution (optional: also add ~100 µL of oxygen powder) to a plastic box (e.g., a pipette tip box that is 13-cm × 9-cm × 5-cm)

16. Cover the hatching container with Saran wrap or a transparent lid. Leave the container on the benchtop overnight (~16–24 h) at 23°C–26°C.

    A typical hatching rate is >70% by the next morning.

17. On day 1 posthatching, add at least two volumes of system H_2O or reverse-osmosis-treated H_2O to the hatching container. Add the H_2O gently to avoid disturbing the juvenile fish. Feed fish as follows and keep the juvenile fish at 24°C–26°C with the same dark–light cycle as the fish facility (e.g., 12-h:12-h dark:light cycle).
    - For fish hatched in a 35-mm × 10-mm Petri dish, feed ~100 µL of freshly hatched brine shrimp using a plastic transfer pipette.
    - For fish in a 60-mm × 15-mm Petri dish, feed ~200 µL of freshly hatched brine shrimp.
    - For fish in a plastic box, feed ~1 mL of freshly hatched brine shrimp.

18. On each day from day 2 to day 4 posthatching, use a plastic transfer pipette to manually remove all the dead brine shrimp, and replace the lost volume of H_2O with fresh system H_2O. Feed freshly hatched brine shrimp as described in Step 17.

19. On day 4 posthatching, use a wide-opening baby brine shrimp net to transfer four juveniles into a 0.8-L tank (prefilled with system water to about half of the tank) that has both 400-µm and 850-µm filters to prevent juvenile fish, which are small, from escaping from the tank. Leave the tank on the regular rack in the fish facility. Turn on the system H_2O flow to ~22 mL/min (approximately two to three drips of system H_2O per second). Feed ~8 mL of freshly hatched brine shrimp (at a density of ~800-shrimp/mL) twice per day. Confirm proper H_2O flow daily.

    Be sure to choose a fish net with a proper mesh size to transfer the juvenile fish, which are ~0.5-cm-long. We use a wide-opening brine shrimp net, which makes it easier to transfer the juvenile fish.

20. On day 17 to day 18 posthatching, place the juvenile fish into new tanks so that the fish density is two juveniles per 0.8-L tank. Adjust the H_2O flow to be a gentle stream (~100-mL/min; the juvenile fish should be calmly swimming and not struggling to swim). Feed ~8 mL of freshly hatched brine shrimp (at a density of ~800-shrimp/mL) twice per day until Step 21.

    The old tanks used for housing the four juveniles during the last 2 weeks can continue to be used for housing two juveniles for the upcoming weeks. However, remove the excess dead brine shrimp, which often accumulate in the bottom of the tank. Confirm proper H_2O flow daily. Choose a fish net with a proper mesh size, as the juvenile fish are now ~1.5-cm-long.

21. On day 31 to day 32 posthatching, assess whether any of the animals has developed color, which signifies male sexual maturation. If so, transfer the animals to larger tanks (e.g., 2.8-L tank for individual fish if paired breeding will be used; 6- to 10-L tanks for three to five females if group breeding will be used [this tank is referred to as a group tank hereafter]) according to their sex. Use dry feed pellets to feed these animals from this point onward as described in the "Fish Diet" section in the Discussion. Adjust the H_2O flow to a gentle stream (the juvenile fish should be calmly swimming).

    Males have vivid yellow and blue colors, whereas females lack such pigments and the female's anal fin is triangular. Representative images of mature versus immature males and females are shown in Figure 2.

    Alternatively, blood worms can be used to feed fish if dry pellets are not available or if the fish do not eat the dry pellets.

22. Around day 40 posthatching, put the male tank next to the female tank so that the males can see the females through the transparent tank wall while still keeping the males and females in separate tanks. After 1–2 d, proceed with Step 23.

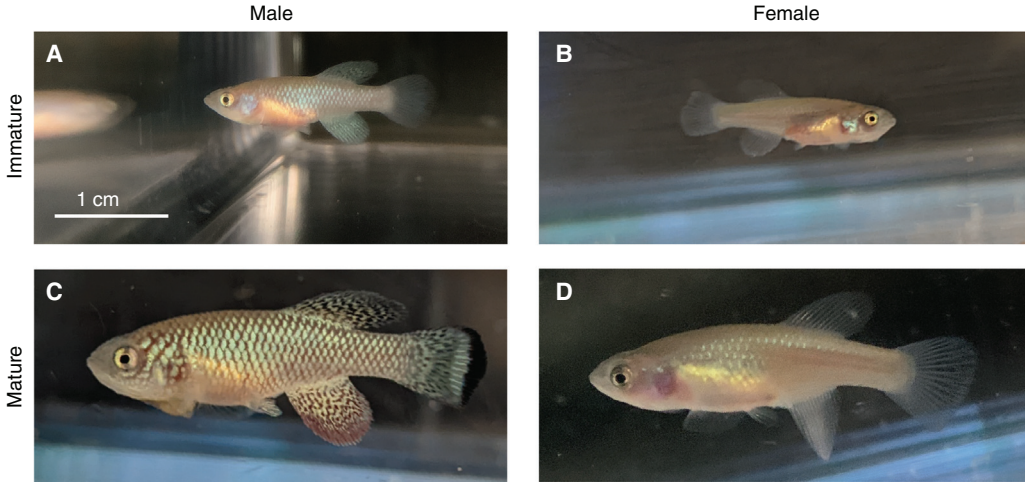


FIGURE 2. *Nothobranchius furzeri* males and females at different developmental stages. (*A*) An immature male (23 d posthatching) right before sexual maturity, beginning to show coloration. (*B*) An immature female (18 d posthatching), which lacks obvious female characteristics at this age. (*C*) A mature, reproductively active male (28 d posthatching) with full coloration. (*D*) A mature, reproductively active female (28 d posthatching) with a round belly and a triangular anal fin.

- For paired mating, alternate the male and female tanks for tank placement.

Place males chosen for group breeding in the group tank with the females or maintain them separately in an individual tank. If we cohouse males and females, we typically cross the animals (Step 23) 1–2 d after placing them in the same tank. Using a 2:1 or 3:1 female to male ratio can reduce the likelihood of male aggression toward the females (see the Discussion for more details).

Cross Setup

23. Feed all animals ~30 min to 1 h before setting up the cross.
    - For paired breeding, feed each adult fish 15–18 mg of the filtered dry feed pellets.
    - For group breeding, dispense 15- to 18-mg increments of dry feed pellets until the fish become disinterested in the food, after the food has been available for >5 min (see the Discussion for details).

    The amount of food required to feed a group tank can be variable, as some of the fish may outcompete others for food.
24. Before breeding, prepare a sand tray by adding 30–45 mL of autoclaved sand to a clean weigh boat (referred to as "sand tray" hereafter).

    This protocol uses sand as a breeding substrate. Peat and glass beads are also viable alternatives that others have used successfully (Blažek et al. 2013; Polačik et al. 2016).
25. Fill the sand tray with clean system H_2O to the brim and remove floating sand particles, either by pouring out some liquid or tapping the surface so that the sand particles sink.
26. Carefully place the sand tray at the bottom of the female tank, being careful not to spill the sand out of the sand tray onto the tank floor.
27. Transfer the male fish into the female's tank if the male is not already there.

    We recommend using a soft, fine mesh fish net for gentle handling of the male.
28. Leave the pair together for the desired time.

    We typically leave the fish together for 4–8 h (see the Discussion for more details). After getting accustomed to mating, the pair will typically start mating minutes after the male is transferred into the female's tank. It is important to observe whether the male shows aggressive behaviors toward the female at this stage. In some cases, we have observed that an aggressive male can kill his mate.
29. After the desired period of mating, remove the sand tray from the tank. For paired mating, transfer the male back into its respective tank.

    The sand tray will contain eggs.

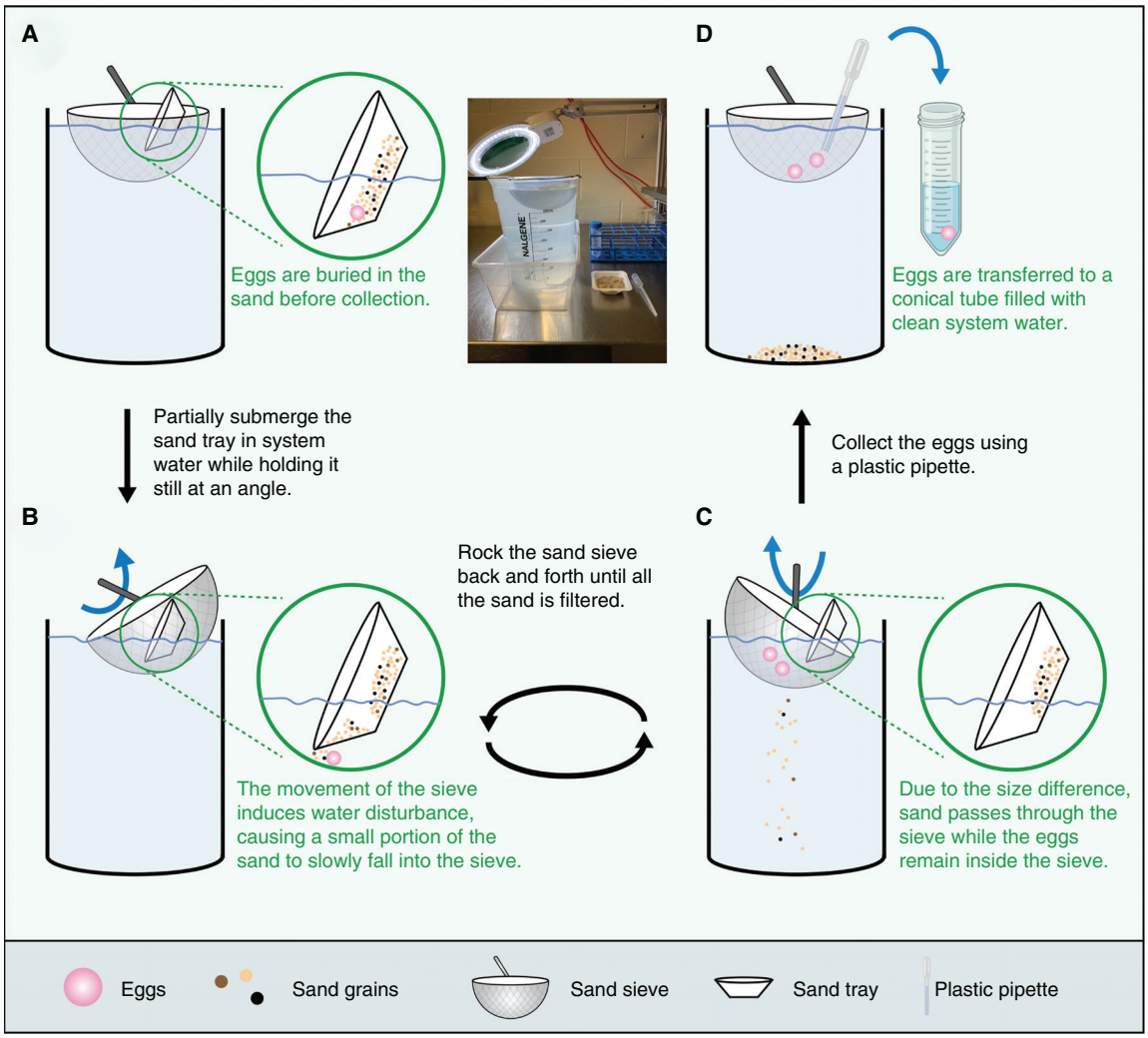
Embryo Collection

In the collection stage, try to handle the embryos as gently as possible because rough handling can result in damage to the embryo's surface. Here, we describe a method of sieving that has worked well for us.

30. Place a 4-L plastic container in a plastic tub and fill the container with clean system H_2O.

    The tub is used for catching any H_2O that might spill over during collection.
31. Shine a lamp over the plastic container to help identify the embryos in the sieve.
32. Hold the sieve so that most of the sieve mesh is submerged in the H_2O.
33. Partially submerge the sand tray in the H_2O over the middle of the sieve and angle the tray with the sand facing toward you (Fig. 3, step A).
34. Holding the tray static over the middle of the sieve, move the sieve toward and away from you in a gentle "U"-shaped motion, so that the motion of the H_2O gently washes the sand out of the sand tray (Fig. 3, steps B–C). Do not dump the entire tray of sand into the sieve as this may damage the embryos. Instead, rotate the sand tray as you wash the sand out, until all the sand has been washed out of the tray. Adjust the angle of the lamp overhead so that the eggs can easily be spotted in the sieve.

    The sand grains are smaller than the sieve holes, so they will pass through the sieve while the eggs, which are larger than the sieve holes, remain inside the sieve.



Note: Objects are not drawn to scale.

FIGURE 3. A gentle method to collect *N. furzeri* eggs. Step A: Completely fill a 4-L plastic container with system H_2O, place the sand sieve inside the container, and orient the sand tray as depicted (a real-life setup is shown to the *right* of the schematic). Steps B–C: While holding the sand tray in place at an angle, rock the sand sieve back and forth as depicted (following a U-shaped track). The movement of the sieve creates gentle waves inside the container, causing a small portion of the sand to fall into the sieve. Because the sand grains are smaller than the sieve holes, they will pass through the sieve while the eggs, which are larger than the sieve holes, remain inside the sieve. After one section of the sand has completely passed through the sieve, rotate the sand tray to submerge the other sections of the tray until all the sand has been processed. Step D: Use a plastic transfer pipette to transfer the eggs from the sieve to a 50-mL conical tube that is filled with clean system H_2O.

35. Use a plastic transfer pipette to transfer the eggs into a 50-mL conical tube (Fig. 3, step D).

    Be careful not to crush the eggs against the sieve with the pipette.

36. After transferring all the eggs into the tube, remove as much of the system H_2O from the 50-mL tube as possible with a transfer pipette. Add ~10 mL of clean system H_2O.

    Using a 50-mL tube minimizes embryo disturbance during transport.

37. After collection, drain the H_2O from the 4-L plastic container and collect the used sand. Store the used sand so that it can be autoclaved and reused in future breeding.

38. Add ~5 mL of fresh embryo solution to a clean Petri dish. Use a 35- × 10-mm Petri dish for <50 eggs.

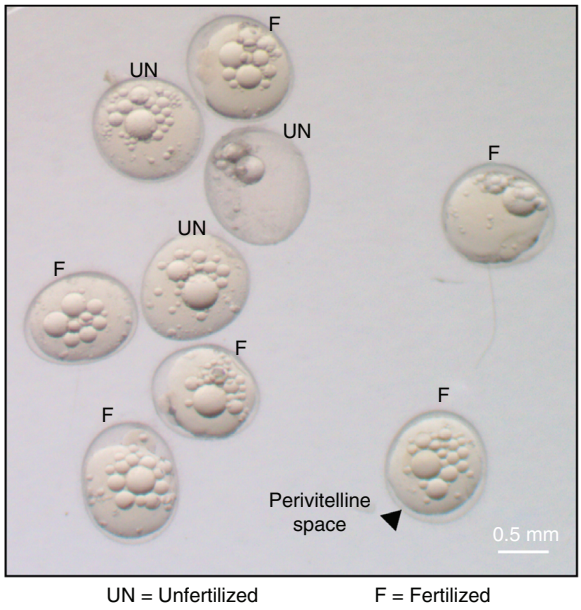


FIGURE 4. Fertilized eggs (F) versus unfertilized eggs (UN). Fertilized eggs have a clear perivitelline space (arrowhead), which is a transparent space between the chorion and the yolk. Unfertilized eggs lack the perivitelline space.

39. Transfer the eggs from the 50-mL tube into the clean Petri dish with a transfer pipette. Transfer as little of the system H_2O as possible.

    Typically, if one waits for the eggs to sink to the bottom of the transfer pipette, one to two drops of liquid will suffice for transferring all the eggs.

40. Stir the dish ~10 times to rinse the eggs.
41. Add ~5 mL of fresh embryo solution to a second clean Petri dish.
42. Transfer the eggs into the Petri dish in Step 41 with a transfer pipette while limiting the amount of liquid transfer as described in Step 39.
43. Stir the dish ~10 times to rinse the eggs.
44. Add ~5 mL of fresh embryo solution to the final clean Petri dish.
45. Transfer the eggs to the final Petri dish while limiting the amount of liquid transfer as described in Step 39.
46. Remove unfertilized eggs, which lack the perivitelline space (Fig. 4, UN).
47. Incubate the Petri dish in an incubator set at a specific temperature, often ranging from 26°C to 30°C.

    A higher temperature would allow more embryos to escape diapause.

Embryo Maintenance

Day 1 to Day 14 after Collection

Perform daily embryo maintenance as described.

48. Use an autoclaved glass pipette to remove dead embryos, which are characterized by the lack of perivitelline space and are stained blue in the presence of methylene blue.

    Dead embryos are morphologically similar to the unfertilized eggs shown in Figure 4.

49. Replace all of the embryo solution with fresh embryo solution using a plastic transfer pipette. Place the Petri dish back into an incubator set at a range of 26°C–30°C (same temperature as Step 47).

50. If fungus develops in the Petri dish or on the surface of the chorion (typically after 3–4 d postcollection), incubate the embryos with a mild iodine solution (12–25 ppm) for up to 2 min, and wash once with ~5 mL of embryo solution. Place the embryos in the fresh embryo solution in a new Petri dish and put the dish back to the incubator.

After 14 d of Incubation

At this point, the embryos should have either entered or escaped diapause (i.e., be developing). Embryos in diapause are identified by the presence of somites and the lack of eyes (Fig. 1C). These embryos can be stored for at least 5 mo at 20°C or lower. The embryos that have escaped diapause should have developed eyes, with clear heartbeats and blood flow (Fig. 1D). These developing embryos can be placed on moist, autoclaved coconut fiber to further develop before hatching. We typically transfer the embryos on coconut fiber as described in this section.

51. Pack a 60-mm × 15-mm Petri dish with coconut fiber using a pair of flat broad-tipped forceps (disinfected with 70% ethanol before use). Press down on the coconut fiber to ensure that the fiber is packed tightly.

    The coconut fiber should fill the whole Petri dish up to approximately three-quarters of the dish height (~1 cm).

52. Place embryos on the coconut fiber.
    i. Fill a glass pipette with some embryo solution before pipetting up the embryo.

       This trick allows the embryo to be released from the glass pipette more easily.

    ii. Use the glass pipette to draw up embryos one at a time.

    iii. Place the embryos ~5 mm apart on the coconut fiber, limiting the amount of liquid transferred to the fiber.

       Transferring excess embryo solution can make the coconut fiber too moist, which can cause the embryos to attempt hatching on the fiber. These embryos could die on the coconut fiber, and fungus may grow around the dead embryos. Placing the embryos ~5 mm apart reduces the chance of any fungus affecting the surviving embryos.

53. Cover the dish with a plastic lid. Place the dish in an enclosed secondary container (e.g., a pipette tip box). Inside this container, place a lidless Petri dish filled with some H_2O.

    This setup helps maintain the moisture of the coconut fiber during the incubation.

54. Place the secondary container for ~2 wk in a 26°C–27°C incubator.

55. Monitor the progress of eye development to determine when to hatch the fish.

    In our experience, the embryos are typically ready to hatch after incubating them on coconut fiber for 14 d (see Step 14 for detailed discussion about when to hatch the embryos).

56. After the embryos become ready to hatch, one can wait for up to ~1 wk before the hatching efficiency drops.

    Hatching efficiency drops if the coconut fiber becomes too dry. Experimenters should try to empirically optimize the level of wetness for the batch of coconut fiber, but in our experience, a few attempts should suffice to develop an intuition about how wet the coconut fiber should be.

57. Proceed with Step 14.

DISCUSSION

Below, we discuss a few additional parameters that one should consider when breeding this fish species.

Paired versus Group Breeding

Breeding can be performed in a paired or group manner. In a paired breeding setup, one male and one female are cohoused during mating. In a group breeding setup (often referred to as "social tanks"),

three to five females are cohoused with one male in a smaller tank (6- to 10-L tank) or eight to 10 females with two to three males in a larger tank (10- to 20-L tank). Group breeding has been reported to yield larger clutches (Žák and Reichard 2021) and thus is routinely used for maintaining fish lines and obtaining embryos for injection. Paired breeding is used when the exact parental information is desired, such as to produce sibling animals that either harbor a genetic mutation or not. Pair-to-pair variations occur in the clutch size; some couples produce >50 embryos per mating, whereas others produce none. The clutch size obtained each week is also variable for the same couple. Therefore, it is advised to set up at least five to 10 pairs of animals to ensure >50 embryos per mating can be obtained for downstream processing.

Fish Diet

We typically feed each adult fish a total of ∼35 mg of dry feed pellets twice per day (∼18 mg per feeding) on the weekdays and ∼35 mg once per day on the weekend, based on our laboratory capability. Feedings on the weekdays are spaced ∼5 h apart—once in the morning and once in the afternoon. The fish for paired breeding are fed when they are individually housed to ensure that all fish receive an equal amount of food. For group breeding, we also feed each group tank twice daily and adjust the total amount of feed if we notice changes in appetite. We will feed more food if the fish display signs of hunger—for example, when the fish actively search for food at the bottom of the tank or near the surface where the food is usually dispensed. Multiple feedings throughout the day can also promote egg production. However, because group tanks have a higher fish density and waste production, H_2O quality in the group tanks can deteriorate quickly if not maintained properly. Thus, we check daily to ensure that H_2O is being cycled out, as well as remove any debris that is causing backup or overflowing.

Notably, different feeding regimens have been described in other protocols, varying in the amount per feeding, frequency of feedings, and the type of food (e.g., supplementing or replacing dry feed with live feed). We refer the readers to several papers for detailed discussion on this matter (Genade et al. 2005; Polačik et al. 2016; Dodzian et al. 2018; Žák et al. 2020, 2022).

Male Aggression

Some males have the potential to be more dominant and aggressive than other fish (males or females). The dominant males can stress, injure, and even kill other members of the same tank. Thus, fish housed in a group should be monitored for signs of male aggression. An aggressive male often chases and nips other fish. Fin damage can indicate that a fish has been a target for aggression, and the submissive fish often darts away from the aggressor.

When signs of aggression have been observed, an aggressive individual should be separated from other fish or replaced by a less aggressive male. Aggressive males tend to be larger than their submissive counterparts, so pairing fish of similar size can reduce instances of aggression. In our experience, we also observed higher mating success and less individual aggression when we kept juvenile fish in a group setting and fed them excess food. Other groups have reported a potential intervention to isolate the aggressive male during the major growth period (4–5 wk posthatching) before reintroducing them back to the communal tanks (Žák et al. 2020). Lastly, using a higher female-to-male ratio when setting up the breeding tank can also reduce male aggression (Žák et al. 2020).

Cross Duration

The cross duration is defined as the time for which the males and females are cohoused in the presence of sand trays. In the literature, different cross durations have been reported, ranging from 2 h to 1 wk (Dodzian et al. 2018). When one is breeding the animals for embryo injection, a shorter cross duration (e.g., 3–4 h) is often used to capture as many embryos in the same developmental stage as possible (it often takes ∼3 h for a fertilized egg to reach the one-cell stage). In our experience, crossing the animals

for 4–8 h yields most of the embryos that would be produced for a given day, and a longer cross is not necessary.

Cross Frequency

Various cross frequencies have been reported in the literature, ranging from daily for ~1 wk (Api et al. 2018), to once per week (McKay et al. 2021), to twice per week (Žák et al. 2020; Žák and Reichard 2021), to continuously cohousing males and females (Dodzian et al. 2018; Hu et al. 2020). Researchers of one paper reported that females had health issues if they were crossed only once per week (Polačik et al. 2014) and recommended crossing the females twice per week. Nevertheless, no systematic study has been published so far in terms of how crossing frequency affects the health of the breeders and their embryos. We typically cross the animals twice per week.

Embryo Collection

Embryos are buried in the sand during mating, so one needs to sieve the embryos out of the sand to collect them. Embryos are fragile immediately after fertilization; their chorion is prone to damage when the sand scratches the chorion surface during collection. Over the next 24 h, the chorion of the embryos hardens, offering better protection from damage. Therefore, incubating the embryos overnight is recommended in the literature (Polačik et al. 2016). However, we sometimes observe fungus around the embryos after the overnight incubation, whereas we rarely encounter this problem when we collect the embryos on the same day. In addition, a same-day collection is needed for embryo injection and reporting fertilization rate (i.e., counting the number of embryos at the one-cell stage). In our experience, one can bypass the overnight incubation by adopting a gentle collection method (Fig. 3) and waiting ~1 h after fertilization before collection (chorion hardens substantially during this hour). Note that others in the field have used different setups successfully, such as embedding a mesh (0.15- to 3-mm hole size) in a container, which allows eggs to be spawned through that mesh directly into the plastic container for collection (see Polačik et al. 2016 for an example setup).

Embryo Maintenance

After the embryos are collected, it is important to replace the system H_2O with fresh embryo solution (or autoclaved system H_2O [Dodzian et al. 2018]) immediately. We recommend several rounds of rinsing and H_2O replacement. Remove dead and unfertilized embryos and replace H_2O daily at least for the first 4 d after embryo collection, a period when most embryo death occurs. If fungus begins to develop around the embryos 3–4 d postcollection, one can treat the embryos with mild iodine or bleach (Polačik et al. 2016; Dodzian et al. 2018). However, one should be cautious about how embryo disinfection affects the health of the embryos and subsequent adults. Additionally, in this protocol, we incubate the embryos in embryo solution, which has methylene blue, for 2 wk before transferring them to incubate on coconut fiber. Methylene blue offers embryos protection from fungus, parasites, and microbes, but according to some reports, overexposure might cause long-term physiological changes (Dodzian et al. 2018). Alternatively, one could switch to incubation in sterilized H_2O after a brief period of incubation in methylene blue (Polacik et al. 2016). In terms of dry incubation substrate, peat moss can be used in place of coconut fiber. Others have also successfully incubated embryos in or on top of peat entirely, without the initial period of incubation in liquid (Polacik et al. 2016). However, some have reported that humid peat substrates allow *Mycobacterium* survival, which could pose a higher risk of infection when embryos are incubated and hatched (Dyková et al. 2021). To reduce the risk of infection, any choice of incubation substrate should be properly sterilized before use.

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).

Mild Iodine Solution

Dissolve two Ringer's tablets (Millipore 96724) in 1 L of MilliQ-purified H_2O or reverse-osmosis-treated H_2O. Dilute a povidone–iodine solution (10% [w/v], 1% [w/v] available iodine; RICCA 3955-16) in the Ringer's solution at the final concentration of 12–25 ppm iodine. Confirm the iodine concentration with an iodine test strip (Fisher Scientific S72405). Protect the solution from light and leave at room temperature during use. Prepare fresh before use.

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