

Protocol

# Life Span Assessment in the African Turquoise Killifish *Nothobranchius furzeri*

Claire N. Bedbrook,<sup>1,2,4</sup> Ravi D. Nath,<sup>1,4</sup> Rogelio Barajas,<sup>1</sup> and Anne Brunet<sup>1,3,5</sup>

<sup>1</sup>Department of Genetics, <sup>2</sup>Department of Bioengineering, <sup>3</sup>Glenn Laboratories for the Biology of Aging at Stanford, Stanford, California 94305, USA

The African turquoise killifish (*Nothobranchius furzeri*) is the shortest-lived vertebrate bred in captivity, with a median life span of 4–6 mo. Within its short life span, the killifish recapitulates critical aspects of human aging, including neurodegeneration and increased frailty. Developing standardized protocols for life span assessment in killifish is critical for identifying environmental and genetic factors that impact vertebrate life span. A standardized life span protocol should have low variability and high reproducibility, and it should enable comparison of life spans between laboratories. Here, we report our standardized protocol for measuring life span in the African turquoise killifish.

## 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)

Coconut fiber (Eco Earth Coconut Fiber EE-8)

*Fill a beaker with commercially available coconut fiber. Add reverse-osmosis H<sub>2</sub>O to the beaker and mix until all the fiber is completely wet. Autoclave the beaker for 1 h on a liquid cycle (~16 psi, 121°C), and then let the coconut fiber cool for about 24 h. Squeeze excess H<sub>2</sub>O out of the cooled coconut fiber and package into reusable plastic containers (e.g., pipette tip or freezer boxes). This does not need to be done aseptically. Store at room temperature. Once opened, use a box of prepared coconut fiber within 1–2 mo; otherwise it becomes too dry.*

Dry fish pellets (Otohime fish diet, Reed Mariculture, Otohime C1)

Hatching solution for killifish <R>

Killifish (males and females for breeding; see Step 1)

Killifish embryo solution <R>

Reverse-osmosis H<sub>2</sub>O

<sup>4</sup>These authors contributed equally to this work.

<sup>5</sup>Correspondence: [abrunet1@stanford.edu](mailto:abrunet1@stanford.edu)

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

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Salt (Instant Ocean, SS1-200)

Sand, autoclaved (The Home Depot, Cemex 200000278)

*Put sand in an autoclave-safe container and autoclave for 1 h on a liquid cycle (~16 psi, 121°C), and then let the sand cool for ~24 h.*

## Equipment

Air pump (Hydrofarm AAPA25L)

Aquatic system (Aquanearing)

Artemia collecting net (Amazon B09FL6L9WR)

Beakers (2-L, 4-L)

Camera (see Step 16)

Dissecting microscope (Leica M60)

Filter bag (Aquanearing MFVB025C)

Filter screens (400- and 850-micron; Aquanearing 400-ZT080S400, 850-ZT080S850)

Fish feeder (Danio Lab, ZF ONE Zebrafish Feeder)

Fish net (Pentair, Aquarium Net)

Hatchery cone (5-gallon; Dynamic Aqua Supply CCH10)

Incubator with 17°C–40°C temperature range (Thermo Scientific Heratherm 50125590)

Labeling tape (ChromaLabel ACAL03800)

Light source (bright enough to visualize embryos; Step 3ii)

Pasteur pipette (glass; Fisherbrand 701865)

Pasteur pipette (plastic; Globe Scientific 138090)

PCR plates (48-well; BIO-RAD MLP4801)

Petri dishes (35-mm and 60-mm; Fisher Scientific 07-000-327 and 07-000-328)

Pipette pump pipettor with thumb wheel (Bel-Art Products F37898-0000)

Plastic tubing (Advanced Technology Products B00E6BCV0G)

Ruler

Scissors (Bonn scissors RS-5840)

Sieve (fine-mesh strainer; OXO 38891)

Tanks (0.8-L, 1.8-L, and 6-L, Aquanearing ZT080, ZT180, and ZT600, respectively)

Tweezers

Wash bottle (BIPEE 500-mL, Amazon B014H428K4)

Weigh boats (85-mm-length × 85-mm-width × 24-mm-depth; Heathrow Scientific 120710)



## METHOD

### Embryo Collection, Hatching, and Raising Fry

*For a life span cohort of 30 male and 30 female animals, collect at least 150 embryos. We recommend harem breeding, whereby one male can breed with three to four females, because this facilitates the collection of large clutches of embryos in a short time period. Typically, obtaining 150 embryos from one male and three to four females will require at least 3 d of collection. For each life span cohort, all collections should be within a one-week time frame to minimize batch effects.*

See Protocol: *Husbandry of the African Turquoise Killifish Nothobranchius furzeri* (Nath et al. 2023) for further details on killifish husbandry and embryo collection.

1. Set up breeding pairs by combining one male and three to four females in a 6-L tank.

*Both male and female breeders should be between 2 and 3 mo of age to obtain the maximal number of live embryos and to minimize potential variability coming from the parents.*

2. Fill a weigh boat halfway with autoclaved sand to create a sand tray. Place the sand tray on the bottom of the breeding tank. Leave the sand tray in the breeding tank for 24 h.

3. After breeding for 24 h, collect the sand tray from the tank and isolate the embryos from the sand as follows (see Fig. 2 in Protocol: Husbandry of the African Turquoise Killifish *Nothobranchius furzeri* [Nath et al. 2023]):
  - i. Fill a 2-L beaker with aquatic system water nearly to the brim. Place a fine-meshed sieve over the beaker so that the mesh of the sieve is in the system water.
  - ii. Pour the contents of the sand tray into the sieve. Sand should pass through the sieve while embryos remain trapped by the mesh. Shine a bright light over the sieve to see the embryos and gently shake the sieve to separate the sand (harsh shaking can damage embryos).
  - iii. Use a plastic Pasteur pipette to pick up individual embryos and place them in a fresh, dry Petri dish.
4. Under a dissecting microscope, identify healthy embryos that have a fully formed chorion (see Fig. 3 in Protocol: Husbandry of the African Turquoise Killifish *Nothobranchius furzeri* [Nath et al. 2023]). Use a glass pipette and a pipette pump pipettor with a thumb wheel to gently aspirate the embryos into the pipette and then gently dispense them into a Petri dish. Use a 35-mm Petri dish for <30 embryos or a 60-mm Petri dish for up to 50 embryos. For collections with >50 embryos, divide the collection into multiple Petri dishes.
5. Remove residual solution from the collected embryos using the pipettor (so the embryos are briefly left dry). Gently add fresh embryo solution from a wash bottle until all embryos are submerged.

*Embryos should not be left dry for prolonged periods of time (>1 min). To reduce the likelihood of contamination, embryo density should be ≤50 embryos per 60-mm Petri dish.*
6. Place the Petri dish containing clean embryos in an incubator maintained at 26°C–27°C.

*At this temperature, most embryos will escape diapause and develop eyes within a 2-wk window.*
7. Maintain killifish embryos in embryo solution for 2 wk.
  - i. For the first four days after collection, transfer healthy embryos to a fresh Petri dish and replace embryo solution with fresh solution daily.
  - ii. Thereafter, inspect embryos daily. Remove dead embryos to prevent contamination. Wash and refresh embryo solution every other day.

*See Troubleshooting.*
8. After development for 14–16 d in embryo solution, transfer the embryos onto coconut fiber in a 35-mm Petri dish as follows:
  - i. Fill a 35-mm Petri dish to half its depth with autoclaved coconut fiber. Press the coconut fiber down to create a flat surface on which to put the embryos.
  - ii. Transfer embryos from solution onto the coconut fiber surface using a glass pipette. Ensure that the embryos are spaced out on the surface of the coconut fiber, with no embryos touching (≤50 embryos per 35-mm Petri dish).
  - iii. Add embryo solution to make sure the coconut fiber is moist.
  - iv. Place the embryos back into the incubator maintained at 26°C–27°C.

*Embryos can be transferred any time between 14–16 d of development; we have not found that there is an optimal time. Embryos that have developed will have large black eyes. If embryos have not developed large black eyes by day 14–16 after collection, they should be excluded from the life span experiment.*
9. Maintain embryos on the coconut fiber for 14–20 d. Throughout this period, ensure the coconut fiber is kept moist, by regularly “watering” with embryo solution from a wash bottle, such that the coconut fiber is moist but not wet (approximately every 4 d).

*The time for maintaining embryos on coconut fiber is flexible (between 14 and 20 d) and can be adjusted to suit experimental aims (e.g., larger cohorts). We have observed the best hatching efficiency with a slightly*

*dry coconut fiber (as opposed to embryo solution-saturated coconut fiber). We therefore allow the coconut fiber to dry for 5 d prior to hatching by not “watering” the coconut fiber for the 5 d prior to hatching.*

10. Hatch embryos as follows:

- i. Use tweezers to gently pick up embryos from the coconut fiber and deposit them into a 60-mm Petri dish filled with 10 mL embryo hatching solution.

*Make sure embryos are submerged in the hatching solution and not floating on the surface. Up to 50 embryos can be hatched in a single 60-mm Petri dish.*

- ii. Leave embryos in hatching solution overnight at room temperature.

*Embryos should hatch within 24 h. For life span experiments, do not use embryos that take longer than 24 h to hatch.*

11. Fill a 0.8-L fry tank containing a baffle, a 400-micron filter screen, and an 850-micron filter screen to prevent fry from being swept out of the tank (Fig. 1A) with aquatic system water. Use a plastic Pasteur pipette to place two hatched fry into one 0.8-L tank (Fig. 1B). Proceed as follows:

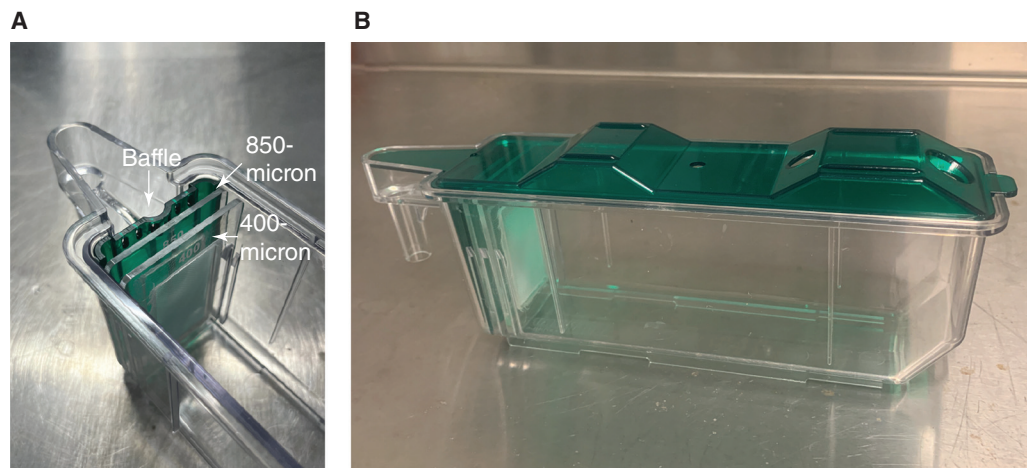
- i. For the first week after hatching, use a slow drip flow rate (~15 mL/min). After the first week, increase the flow rate to a low-pressured flow stream (~110 mL/min).
- ii. Two weeks after hatching, remove the 400-micron filter to allow better system water flow.
- iii. Three weeks after hatching, remove the 850-micron filter to allow better system water flow and more efficient outflow of debris from the tank.

12. Feed fry freshly hatched brine shrimp (*Artemia sp.*) (see Step 13) for the first 4 wk after hatching. For life span experiments, we feed fry two times a day, at 9:00 a.m and 3:30 p.m., 7 days a week. System water flow is essential to wash out residual uneaten food and waste from the tanks. Thus, it is key to regularly check that each fry tank has flow (as described in Step 11). If flow is off, the ammonia level can reach toxic levels. We recommend checking flow daily.

13. Prepare brine shrimp as follows:

### Hatch Brine Shrimp

- i. Fill a 5-gallon hatchery cone with 14 L of reverse-osmosis H<sub>2</sub>O.
- ii. Insert tubing connected to a running air pump (i.e., a bubbler) and make sure it is vigorously bubbling in the H<sub>2</sub>O.
- iii. Add ~230 mL (~1 cup) of Instant Ocean salt.



**FIGURE 1.** Fry tank housing. (A) 0.8-L tank with a baffle and 400- and 850-micron filter screens. (B) Complete 0.8-L tank setup.

- iv. Add ~60 mL (~1/3 cup) of brine shrimp eggs.
- v. Propagate for 2 d at room temperature with constant vigorous bubbling.  
*Brine shrimp will be ready as feed in 2 d.*

### Harvest Hatched Brine Shrimp

- i. Remove bubbler from hatchery cone to allow freshly hatched brine shrimp to settle at the bottom; this takes 5 min.
- ii. Drain the hatched brine shrimp layer into a plastic beaker.
- iii. Place bubbler in the plastic beaker with hatched brine shrimp for aeration.  
*If brine shrimp are left without the bubbler for extended periods of time (~1 h), they will die. Hatched brine should be used within 24 h. As the brine shrimp age, their nutrient profile changes and they are more likely to die and cause the batch to go bad. Using the brine shrimp within 24 h of hatching is optimal to ensure reproducible nutrient content and fresh food.*
- iv. Brine shrimp filtering: before feeding brine shrimp, position 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.
- v. Pour hatched and recently filtered brine shrimp into a 500-mL squeeze wash bottle. Brine shrimp should be filtered within 30 min of feeding. For each feed, squeeze the wash bottle for 3 sec per tank to deposit ~15 mL of brine shrimp into each tank.  
*The feeding regimen (quantity and regularity) is critical to reproducible life span measurement. As with other animals, if killifish are underfed, they exhibit life span extension (Terzibasi et al. 2009; Žák et al. 2020).*

### Adult Tank Housing

Four weeks after hatching, adult, sexually mature killifish should be individually housed in 1.8-L tanks (Fig. 2A). At 4 wk, animals should be sexually mature, with males showing strong caudal fin coloration. Individual animals that are developmentally delayed and not sexually mature at 4 wk are not included in life span cohorts. At this point, we recommend photographing fish with a ruler to track the size of the fish as this can ensure that different cohorts have matured at the same rate and can identify differences in growth phenotypes between cohorts (e.g., due to different genotypes).

14. Fill 1.8-L tanks (fitted with a 1.8-L tank baffle) with 1 inch of aquatic system water (Fig. 2).



**FIGURE 2.** Adult tank housing for life span assessment. (A) Complete 1.8-L tank with lid and baffle with ~1 inch of aquatic system water. (B) Photography station for measuring fish at 4 wk of age. Tank is placed above a flat ruler such that the ruler is imaged through the ~1 inch of system water.

We do not use filters for the 1.8-L tanks because we find they are not necessary and can cause clogging and overflow. The circulating system automatically maintains the following system water parameters: temperature 26°C–27°C, pH 6.5–7.0, and conductivity 3800–4000  $\mu\text{S}/\text{cm}$ . Note that killifish are fine in system water with a wide range of conductivity (650–4000  $\mu\text{S}/\text{cm}$ ) (Dodzian et al. 2018; Astre et al. 2022). Higher conductivity can inhibit velvet disease (caused by parasites) (Astre et al. 2022). These parameters should be assessed daily. Killifish are maintained with a 12:12 h light-dark cycle, with light turning on at 7 a.m. and off at 7 p.m. Regular checks and maintenance of the system are critical. In our experience, the pH meter and pumps of the recirculating system are the components that fail most often. We keep a backup stock of these items.

15. Transfer each 4-wk-old fish into a prepared 1.8-L tank (one fish per tank) using a net.
16. Place the tank on a ruler for scale, and with a camera (e.g., iPhone), take an overhead photograph of the tank. Do this for every fish.

*These images can be analyzed to determine young adult fish size by simply reading off the length of the fish against the ruler. It is critical to take the photo on a level surface with a consistent amount of system water in each tank (~1 inch of aquatic system water; Fig. 2B). The use of a relatively shallow liquid level (~1 inch) reduces potential variability of fish height in the tank. If the fish is higher in the tank, the fish will be closer to the overhead camera and appear larger than if it is lower in the tank. Hence, it is critical to use a consistent level of shallow system water for these photographs. It is also critical to include a ruler under the tank for each picture to account for any changes in the imaging setup.*

17. Provide each tank with an appropriate identification tag detailing the fish and hatch data (e.g., wildtype-1 hatched: 1/28/22).
18. Place each tank on the system with a continuous low flow of system water (~300 mL/min).

*A rapid flow rate can stress the fish. However, too low a flow rate results in poor H<sub>2</sub>O quality within the tank. A low-pressure steady stream is ideal.*

19. Feed adult fish dry food pellets (Otohime diet) three times a day on weekdays (at 9:00 a.m., 1:30 p.m., and 3:30 p.m.) and one time a day on weekends (12:00 p.m.). Use the ZF ONE zebrafish feeder to deliver 20–30 mg of Otohime fish diet into each tank per feed.

*It is crucial that all the food makes it into the tank and does not fall on the lid.*

*This feeding regimen provides a total of ~60–90 mg per day on weekdays and ~20–30 mg per day on weekends, for a total of ~340–510 mg per week. Note that this is more than our maintenance feeding (which is ~240–360 mg per week).*

*The feeding regimen (amount and type of food, and spacing between feedings) is a major source of life span difference between protocols. With the feeding regimen outlined in this protocol, we observe a reproducible median life span ranging from 145 to 160 d for male GRZ strain killifish raised in our system at a temperature of 26°C–27°C.*

*Note that this feeding regimen differs from other feeding regimens our laboratory has used previously, which range from 1/32 teaspoon of dried blood worms (Hikari Inc. 33210) two times a day on weekdays and once a day on weekends (Hu et al. 2020) to 245 mg of Otohime diet per week using automated feeders (McKay et al. 2022).*

20. Check fish health daily.
21. Check tank liquid level daily to ensure the fish are getting the appropriate flow rate.

*If fish display signs of non-age-associated illness (e.g., clear sign of infection or wound), the fish should be removed from the system, euthanized, and removed from the life span cohort.*

*The flow rate should not be impeded. See Troubleshooting.*

22. Monitor fish until death. At the time of death, do the following:

- i. Record death date with genotype and hatch date.
- ii. Remove dead fish from tank and photograph with a ruler to enable assessment of fish size at death.

*It is critical to include a ruler for each picture to account for any changes in the imaging setup.*

- iii. Take a small tail biopsy and place it in a designated well of a 48-well polymerase chain reaction (PCR) plate (recording well location for each fish) and store at –20°C.

*Tail clips can be genotyped to confirm fish genotype if multiple genotypes are being compared in the life span experiment.*

## TROUBLESHOOTING

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**Problem (Step 7):** Embryo death from fungus or parasites is observed.

**Solution:** It is possible to disinfect embryos with iodine or bleach (Dodzian et al. 2018; Astre et al. 2022). However, to our knowledge, the impact of such treatments on life span has not been tested.

**Problem (Step 21):** Inappropriate system water flow is observed.

**Solution:** If the tank liquid level is low, the flow from the inlet tube might be blocked, causing rapid dirtying of the tank. This can be fixed by increasing the flow rate for the affected tank to flush out any blockage in the tubing. If the tank water level is high, there might be waste buildup at the baffle that could eventually lead to an overflowing tank; clean the baffle.

## RELATED INFORMATION

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It is important to note that husbandry parameters (such as food type, amount, and feeding frequency) are major factors in life span variability between labs. Further information regarding other standardized protocols for assessment of life span in killifish and comparison of life spans between laboratories can be found in the following references: Polačik et al. (2016); Dodzian et al. (2018); Hu et al. (2020); Žák et al. (2020); and Astre et al. (2022).

## RECIPES

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### *Hatching Solution for Killifish*

Dissolve 1 g of humic acid (Sigma-Aldrich 53680) in 1 L of reverse-osmosis-treated H<sub>2</sub>O. 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<sub>2</sub>O or reverse-osmosis-treated H<sub>2</sub>O. Sterilize the solution with 0.22- $\mu$ m 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  $\mu$ L of methylene blue). Protect the solution from light, and store at room temperature (good for at least 2 mo).

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