Video Article

A Protocol for Laboratory Housing of Turquoise Killifish (*Nothobranchius furzeri*)

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Abstract

The development of husbandry practices in non-model laboratory fish used for experimental purposes has greatly benefited from the establishment of reference fish model systems, such as zebrafish and medaka. In recent years, an emerging fish – the turquoise killifish (*Nothobranchius furzeri*) – has been adopted by a growing number of research groups in the fields of biology of aging and ecology. With a captive life span of 4 - 8 months, this species is the shortest-lived vertebrate raised in captivity and allows the scientific community to test – in a short time – experimental interventions that can lead to alterations of the aging rate and life expectancy. Given the unique biology of this species, characterized by embryonic diapause, explosive sexual maturation, marked morphological and behavioral sexual dimorphism - and their relatively short adult life span - *ad hoc* husbandry practices are in urgent demand. This protocol reports a set of key husbandry measures that allow optimal turquoise killifish laboratory care, enabling the scientific community to adopt this species as a powerful laboratory animal model.

Video Link

The video component of this article can be found at https://www.jove.com/video/57073/

Introduction

Given their short life span and rapid life cycle, turquoise killifish are rapidly growing as a promising new model organism in biology[1,2,3]. This species is characterized by a unique life cycle for a teleost, consisting of embryonic diapause, rapid sexual maturation, and an extended post-reproductive life stage[4,5]. Recent work has contributed to elucidating the biology of this species both in captivity and in the wild[6,7]. Turquoise killifish live in seasonal fresh water bodies that form during the rainy season in the African savannah in Zimbabwe and Mozambique. During the dry season, embryos survive in the dry mud in the absence of water by virtue of a stress-resistant life stage called diapause.

Genetic maps for this species have been generated[8,9], and recently their genome has been sequenced and assembled[10,11]. Several inbred laboratory fish strains have been developed, and transgenesis and genome editing via CRISPR/Cas9 have become available in this species, *de facto* promoting turquoise killifish as a competitive laboratory vertebrate model organism[12,13,14].

Although a laboratory protocol has already been published for this species[15], in the present protocol we develop a comprehensive list of experimental laboratory guidelines that are specifically aimed at studies that investigate aging and survival. The present protocol enables researchers already familiar with zebrafish and medaka husbandry to become versed in turquoise killifish husbandry by adopting a minimum number of key adjustments. At the same time, this protocol provides researchers without prior experience in fish husbandry with the essential tools to raise a thriving turquoise killifish colony.

Protocol

Fish are raised at 28 °C in a water recirculation system (see Water Parameters), with 10 - 20% daily water disposal. Three different tank sizes are recommended: 0.8 L, 2.8 L, and 9.5 L. Each tank receives a constant water flow of 2 mL/s.

1. Reagents Preparation (Not Included in Materials)

NOTE: African turquoise killifish (*Nothobranchius furzeri*) can be provided from an established laboratory stock. The annual killifish desiccation-resistant embryos can be shipped by mail. It is critical to ship embryos within 8 - 30 °C temperature range.

1. Prepare humic acid (hatching) solution by dissolving 1 g/L humic acid in system water. Autoclave and store at 4 °C for up to 10 weeks.

2. For preparing HUFA Artemia enrichment, add HUFA enrichment to brine shrimp hatcher daily at a concentration 500 µL/L of brine shrimp solution.
3. Prepare methylene blue solution by dissolving 100 µL/L of methylene blue stock solution in previously autoclaved system water. Since methylene blue is light-sensitive, keep the solution in dark bottles, or cover with foil. Store at RT.
4. Prepare coconut fiber as a solid substrate for embryo incubation. Alternatively, use a filter paper (see section 1.5).
    1. Presoak coconut fiber with distilled water. Autoclave and store at 4 °C for up to 5 weeks.
    2. On the day of embryo transfer, prepare Petri dish with moist coconut fiber.
    3. Fill a 90-mm diameter Petri dish with coconut fiber under a fume-hood and next to a flame, to reduce contamination by yeast and bacteria.
    4. Compact coconut fiber to a height of 1 cm, with sterile tissue. Remove most of the moisture from the coconut fiber by pressing a paper towel on top of the plate, letting the paper to absorb the excess water. Heat a metal spoon over the flame, and press down on the entire surface of coconut fiber. This prevents coconut fiber fungal/bacterial contamination.

5. Prepare the filter paper as a solid substrate for embryo incubation.
    1. On the day of embryos transfer, place 3 layers of filter paper disks that fit the 90-mm Petri dish. Add 5 mL of humic acid solution to keep humidity.

2. Breeding

1. **Breeding turquoise killifish for strain maintenance**
   NOTE: Following this protocol, sexual maturity is reached at ~4 weeks post-hatching and fecundity peaks between 7 - 9 weeks. It is critical to note that fecundity depends on feeding frequency and food quality; therefore, at least two feedings a day per breeding tank are recommended to raise embryos yield (see section 5.6.)
    1. Setup a 9.5 L breeding tank. Fill with system water and add one male and two female fish.
    2. As male African turquoise killifish display dominance during mating, which might lead to harassment of females, choose a male with a slightly smaller body size than the female to reduce mating stress and increase reproductive output. Set up 5-week-old males with 6/7-week-old females.
    3. Fill a plastic container (10 x 10 x 5 cm) with autoclaved sand reaching a final depth of ~2 - 3 cm and place the sand box in the center of the breeding tank.
    4. Let turquoise killifish breed continuously and harvest embryos once a week for embryo incubation.
       NOTE: The use of sand substrate poses challenges to centralized filtration systems and should be replaced by alternative methods in the future. Possible alternatives could be the use of zebrafish breeding tanks.

2. **Breeding for transgenesis**
   NOTE: Embryos to use for injections need to be synchronized at the one-cell-stage, and this requires that they are collected immediately following fertilization.
    1. To harvest one-cell-stage embryos to use for injection and generation of transgenic lines, set up a breeding tank with one male and two female fish (same as 2.1.).
    2. Two days prior to embryo collection, isolate the male in an individual tank and keep the male in visual contact with adult females.
    3. On the day of collection, add the male and a sand box to the breeding tank and let them spawn for 2 h.

3. Embryo Husbandry

1. **Embryo collection**
   NOTE: Embryo collection is performed by sieving and harvesting embryos from the sand box. Under normal conditions, each sand box should contain from 30 to 200 embryos.
    1. On the day of collection, remove sand box from the breeding tank. Empty the sand box into a strainer (~0.9 mm strain size) and rinse with system water. This can be done over a large tank to collect sand for autoclaving.
    2. Partially submerge the strainer in system water and swirl gently, letting the embryos to group together in the center.
    3. Collect embryos with a 10 mL Pasteur pipette.
    4. Transfer embryos to a 90 mm Petri dish in ~40 mL of system water.
    5. Inspect embryos in Petri dish under a light stereomicroscope and remove those presenting ruptured chorion and signs of damage.
    6. Proceed directly to **Embryo bleaching**.
       NOTE: Always use one strainer per fish strain in order to prevent potential cross-strain embryo contaminations.

2. **Embryo bleaching**
   NOTE: Embryo bleaching prevents microorganisms present in fish tanks from contaminating the incubation media.
    1. Prior to bleaching, use a disposable Pasteur pipette to remove the system water from the Petri dish containing collected embryos.
    2. To prevent unwanted fungal and bacterial growth, add 50 mL of freshly prepared H_2O_2 (1% v/v in autoclaved system water) to the collected embryos.
    3. Shake embryos for 5 min at a low speed in 90 mm Petri dishes in 50 mL solution.
    4. Remove H_2O_2 solution with disposable Pasteur pipette and wash embryos three times for 5 min with 50 mL of methylene blue solution. Remove methylene blue solution.
    5. Add 50 mL of H_2O_2 (1% v/v in autoclaved system water) to embryos and shake for 5 min.
    6. Remove H_2O_2 solution and wash three times for 5 min with 50 mL methylene blue solution.
    7. Incubate embryos at 28 °C to increase synchronous embryos development, at a maximum density of 100 embryos per 90 mm Petri dish in 40 mL of methylene blue solution.

NOTE: Do not extend embryo incubation in the bleaching solution. This may cause damage to the egg chorion and increase embryo mortality. Embryo bleaching could cause major physical-chemical changes in the egg chorion that could result in altered chorion physiology and hatching success.

3. **Embryo incubation in methylene blue**
NOTE: Liquid incubation in methylene blue solution prevents parasite growth and enables detection of dead embryos and unfertilized eggs.
    1. Inspect incubated embryos, removing any dead embryos (stained blue by methylene blue) from the Petri dish to prevent fungal and bacterial contamination that affect the survival of live healthy embryos.
    2. Remove old methylene blue solution and replace with fresh solution.
    3. Return Petri dish to 28 °C incubator (**Figure 1A**). Within 7 - 10 days, ensure that the developed embryos show visible black eyes. Transfer these embryos to the coconut fiber or the filter paper solid substrate medium (**Figure 1B**).
    4. Retain undeveloped embryos in methylene blue, monitor daily, and transfer to solid substrate medium once black eyes have developed.
    5. Repeat steps 3.3.1-3.3.3 daily until embryos have visible black eyes.
    NOTE: Constant exposure of embryos to methylene blue may induce long-term changes in adult fish physiology.

4. **Embryo transfer to filter paper**
NOTE: Turquoise killifish embryos can develop on a dry substrate, recapitulating natural conditions. Additionally, dry embryo incubation enables researchers to synchronize embryos and hatch them on the same day.
    1. As developed embryos will have visible black eyes within 7 - 10 days, use a disposable Pasteur pipette or fine curved tweezers to transfer embryos from the methylene blue solution onto a previously prepared filter paper plate.
    2. Spread embryos ~5 mm apart with forceps, up to 100 embryos per 90 mm plate (**Figure 1B**).
    3. Seal the Petri dish with parafilm.
    4. Incubate embryos at 28 °C for 2 - 3 weeks, until they have fully developed golden irises and are ready for hatching (**Figure 1C**).
    NOTE: Do not prolong incubation of ready-to-hatch embryos for longer than 2 weeks as their viability will be dramatically reduced.

5. **Embryo transfer to coconut fiber**
NOTE: Autoclaved, sterile coconut fiber (or organic peat moss) can be used as a valid alternative medium for solid substrate incubation.
    1. Use a disposable Pasteur pipette or fine curved tweezers to transfer embryos from the methylene blue solution onto a ready-to-use coconut fiber plate.
    2. Spread embryos ~5 mm apart, up to 100 embryos per 90 mm plate (**Figure 1B**).
    3. Seal the Petri dish with parafilm.
    4. Incubate embryos at 28 °C for 2 - 3 weeks, until they have fully developed golden irises (e.g. in **Figure 1C**).
    NOTE: For long-term storage (up to one year), transfer embryos at 3-days post collection from methylene blue solutions to a solid-substrate plate at 17 °C. Incubate embryos until they develop black eyes.

4. Hatching Turquoise Killifish

NOTE: Turquoise killifish embryos can be successfully hatched in a humic acid solution[14].

1. Using fine curved tweezers, transfer carefully 50 - 100 developed embryos into the hatching box filled with the humic acid solution at 4 °C. The humic acid solution consists of 1 g/L humic acid in system water. Autoclave and store at 4 °C for up to 10 weeks. Make sure that all embryos are completely immersed. The humic acid solution must be shallow, not deeper than 2 cm.
NOTE: Low temperature of humic acid solution improves hatching and complete immersion of the embryos in the solution allows synchronized hatching.
2. Place the hatching box into the 28 °C hatching incubator. Cover the hatching box with the lid. To supply sufficient aeration, connect hatching box by tubing with air supply.
NOTE: Not sufficient aeration during the incubation results in high rates of fry not able to fill the gas bladder ("belly-slider" phenotype, see Note in section 5.1)
3. From the day after hatching, to maintain the adequate water quality in the hatching box, add autoclaved system water once a day in the proportion of 1:1, keeping a final depth of 2 cm.
4. Transfer unhatched embryos back to the solid substrate, and attempt hatching a week later.
NOTE: Upon hatching, turquoise killifish are readily capable to uptake and consume live food. For optimal growth, feed fry twice per day with excess freshly hatched brine shrimp (*Artemia salina*). The sign of full satiation is the orange-colored abdomens of fry after 10 - 15 min of each feeding. Siphon out the excess, uneaten and decomposed brine shrimp using a Pasteur pipette on a daily basis.

5. Raising Juvenile and Adult Fish

1. At five days post-hatching, move juveniles to the water re-circulation system. Using disposable plastic pipettes (or a plastic spoon), carefully transfer five juveniles per 0.8 L tank equipped with 400 µm fry screen (**Figure 2**).
NOTE: It is possible that a portion of juvenile killifish will not have filled the gas bladder, resulting in a typical "belly-slider" phenotype, characterized by fish not reaching proper buoyancy, forcing them to continuously swim, causing severe malformations in adult fish. These fish cannot be used for survival assays or for efficient breeding and need to be censored.
2. Feed juveniles twice per day with freshly hatched brine shrimp in excess until 14 days post-hatching. Siphon out debris from the bottom of each tank daily.
3. At 14 days of age, transfer juvenile fish to 2.8 L tank equipped with an 850 µm fry screen. From this point onwards, label each tank with fish ID, indicating hatch date, strain information, fish gender and fish identification number (**Figure 3**). For survival assays, individually house each fish in single tanks from this point onwards.

4. For the following 7 days, feed juveniles twice per day with ~2 mL of brine shrimp per fish. At this stage fish can be supplemented with 1 - 3 live blood worms (in case the blood worm larvae are too large for the fish, chop them into smaller pieces with a razor blade). To prevent deterioration of water quality, siphon out uneaten food and additional waste twice per week.
5. After 3 weeks from hatching, remove fry screen from the back of the tank and start to feed each fish twice per day ~2mL of brine shrimp and 0.5 mL of blood worm. At this stage, juveniles should have reached 1 cm in body size and should be capable of ingesting full-size blood worm.
6. At 4 weeks of age, feed each fish twice per day with ~2mL of brine shrimp and 1 mL of blood worm. Females can be co-housed at a density of up to 3 females per 2.8 L tank.
7. At this stage ensure that fish reach complete sexual maturation. Check for the presence of large dorsal, anal and caudal fins with signs of coloration in males and round abdomens full of eggs in females.
   NOTE: Raising adult fish in individual tanks for survival cohort studies may negatively affect fish behavior and health. However, group housing for survival cohort studies adds significant confounding factors due to the establishment of social dominance and male territories, leading to strict social hierarchies.

6. Feeding

Note: Laboratory turquoise killifish can be fed a combination of baby brine shrimp (*Artemia salina* nauplii) and blood worm (*Chironomus spp*. larvae). Turquoise killifish fry are fed exclusively baby brine shrimp. Juvenile and adult fish are fed twice a day both brine shrimp and blood worm (**Figure 2**). Ideally, fish can be fed multiple times a day, exceeding the 2 feedings indicated in this protocol.

1. **Culturing brine shrimp**
   1. Add 10 L of reverse osmosis (RO) water and 350 g of red sea salt to a brine shrimp hatcher and dissolve by aeration with an aeration tube.
   2. Enrich the culture with 5 mL of highly unsaturated fatty acid (HUFA).
   3. Add 20 g of brine shrimp cysts into the hatching solution. Inspect that brine shrimp cysts do not float on the surface of the water and ensure proper oxygenation and circulation of the culture.
      NOTE: Daily aliquots of dry brine shrimp cysts can be stored at 4 °C.
   4. On the afternoon of the next day, supply the culture with another aliquot of 5 mL of HUFA.

2. **Harvesting hatched brine shrimp**
   NOTE: After ~36 h from the starting culture, brine shrimp are ready for harvesting (instar II phase).
   1. Collect 5 L of the culture in a container using the tap on the bottom of the hatcher and let sit for 10 min.
   2. After 10 min, remove brine shrimp shells (brown color) from the top of the 5 L container and filter the hatched brine shrimp (orange color) through a mesh. Pay attention to exclude the sediment found at the bottom of the container as it contains non-hatched eggs and dead brine shrimp.
   3. Rinse hatched brine shrimp with RO water into a 2 L container and let sit for 10 min.
   4. After 10 min, filter brine shrimp again through a mesh and collect in 2 L of RO water.
   5. Repeat the previous three steps until brine shrimp solution is free from non-hatched cysts and brine shrimp shells.
   6. Transfer brine shrimp from the 2 L container into squeeze bottles for feeding.
      NOTE: Culturing brine shrimp is fairly robust and reliable. However, to avoid shortage of brine shrimp in case of unsuccessful hatching, smaller (500 mL) backup hatchers can be used.

3. **Setting up the backup hatcher**
   1. Dissolve 17.5 g of red sea salt in 500 mL of RO water by aeration.
   2. Enrich culture with 500 µL of HUFA.
   3. Add 2 g of brine shrimp cysts.
   4. After 18 - 24 h, supply the culture once more with 500 µL of HUFA.
      NOTE: Brine shrimp are ready to harvest after ~24 h.

4. **Preparation of live blood worm**
   1. Immediately prior to feeding, filter an appropriate amount of blood worm through a strainer using RO water.
      NOTE: Live blood worm can be stored at 4 °C for 7 - 10 days.
   2. Rinse blood worm with a small amount of RO water into a plastic container.
   3. With a plastic Pasteur pipette (narrow tip removed), take up the blood worm mixture for feeding.
      NOTE: Feeding laboratory killifish colonies with live food from un-controlled sources adds a risk for external contaminations from parasites and potentially pathogenic microbial communities. In the future, an ad hoc sterile fish feed should be developed.

7. Killifish Laboratory Strain Genotyping

NOTE: To distinguish among turquoise killifish strains, as well to determine sex within each strain, specific genetic (microsatellite) markers can be used[9] (**Table 1**).

1. **Sampling**
   1. Hold the fish securely in a net on the top of wet sponge.
   2. Swab 2 - 3 scales from the fish body from the operculum to the caudal fin using cotton swabs.
   3. Pick scales from the swab and transfer 2 - 3 scales in a 1 mL tube containing NaOH solution (200 µL 0.5 mol/L NaOH, 1% β-Mercaptoethanol, and 0.5% polyvinyl pyrrolidone).
   4. Spin the PCR tubes for 15 s to make sure that scales are completely immersed in the NaOH solution.

2. **Genomic DNA isolation**
    1. Incubate the sample for 20 min at 95 °C.
    2. Cool at RT, neutralize sample with 1/10 volume of 1 M Tris-HCl, pH 8.0.
    3. Centrifuge the sample for 5 min at full speed.

8. Water Parameters

NOTE: Husbandry of organisms whose intended use is adult phenotyping requires highly stable husbandry conditions throughout the life span of the target species. Therefore, culturing water organisms, such as turquoise killifish, necessitates strict control of water parameters. Water recirculation, with additional four-steps water filtration, ensures a robust basis to attain control over water parameters, providing all the tanks with the same water conditions over time. It is recommended to reconstitute the system water from reverse-osmosis (RO) water, added with commercial marine salt and sodium bicarbonate.

1. Water circulation system scheme: First, waste-water from fish tanks flows through solid particles metal filter that captures all un-eaten food debris and larger particles. Metal filters are rinsed three times a week; Second, following the first mechanical filtration, water is conveyed in large sumps and then pumped to a biofilter, where bacteria convert ammonia to nitrites and nitrates; Third, from the biofilter, water is pumped to 25-µm filter sleeves, which trap finer size particles. Finally, water flows through ultraviolet (UV) lamps that sterilize water from bacteria and viruses. Following these four steps, filtered water returns to the fish tanks.
2. To reduce microorganism growth in the tanks, prevent accumulation of nitrates, and reduce overall salinity, 10 - 20% of the system water is disposed on a daily basis.
3. Maintain water temperature constant at 28 °C, water pH constant within the 7.0 to 7.5 range.
4. Although killifish tolerate wide range of salinity, to avoid oodinosis, maintain conductivity within the range 650 - 710 micro-Siemens. A year-long 12 h light/dark cycle ensures colony health and productivity.
   NOTE: Killifish can tolerate water conductivity up to 1500 micro-Siemens.

Representative Results

Turquoise killifish proper husbandry results in median survival ranging between 12 - 18 weeks in the GRZ strain (e.g. **Figure 4A**). Variations of median survival depend on diet, feeding frequency, and housing temperature conditions. Poor husbandry results in survival curves presenting increased early mortality and repetitive, sudden drops of survival throughout time, characterized by several inflection points (**Figure 4B**).

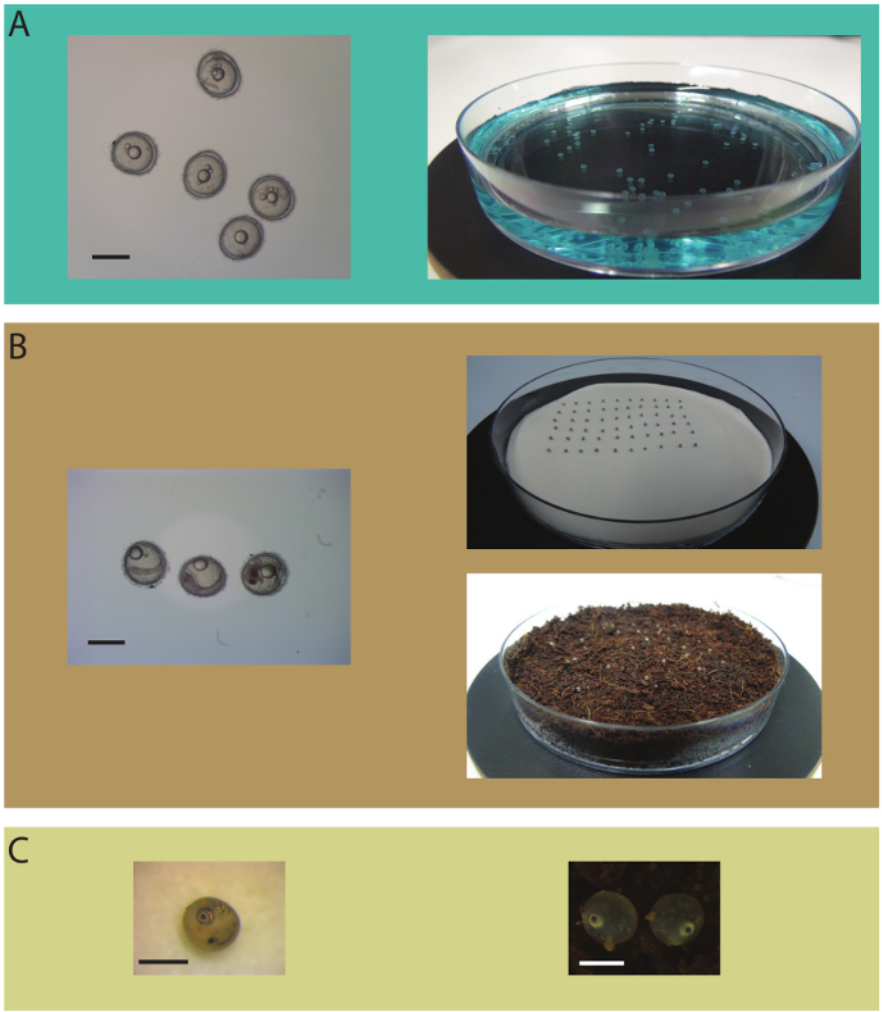

Figure 1: Representative embryonic developmental stages with respective incubation substrate. (A) Freshly collected embryos, incubated in methylene blue solution in the incubator at 28 °C. **(B)** Embryos ready to be transferred to solid medium, either filter paper or coconut fiber. **(C)** Embryos ready to be hatched, displaying typical golden irises. The scale bar is equal to 1 mm. Please click here to view a larger version of this figure.

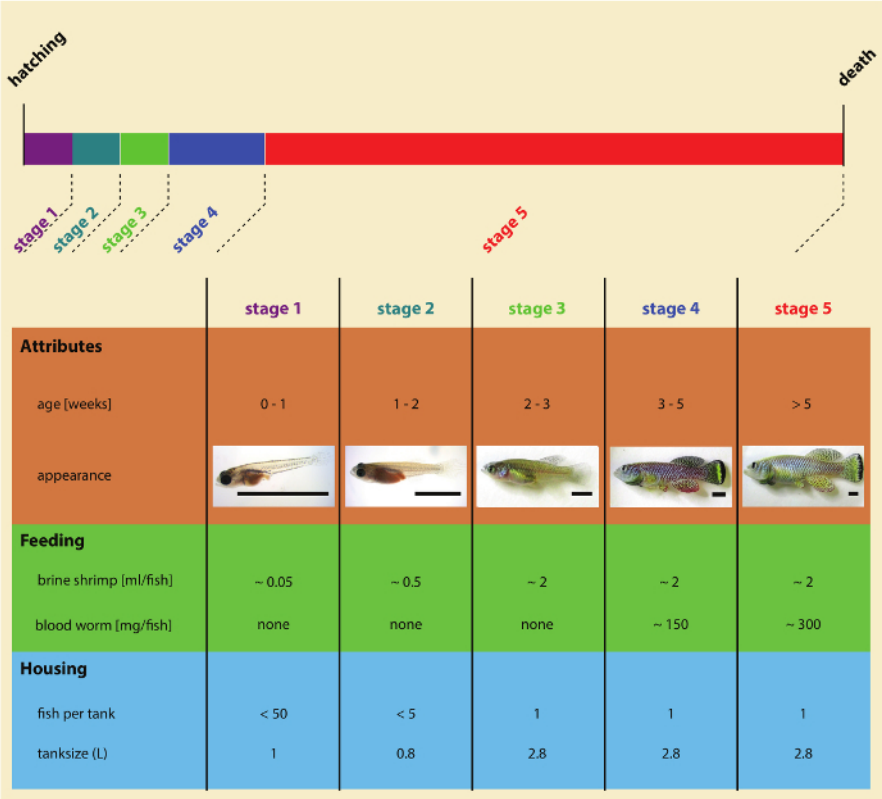

Figure 2: Stages of turquoise killifish post-hatching development. Please click here to view a larger version of this figure.

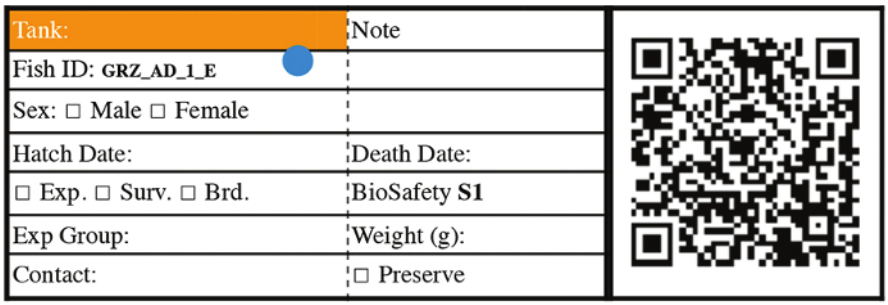

Figure 3: Representative fish ID tag for fish from stage 3 onwards. Please click here to view a larger version of this figure.

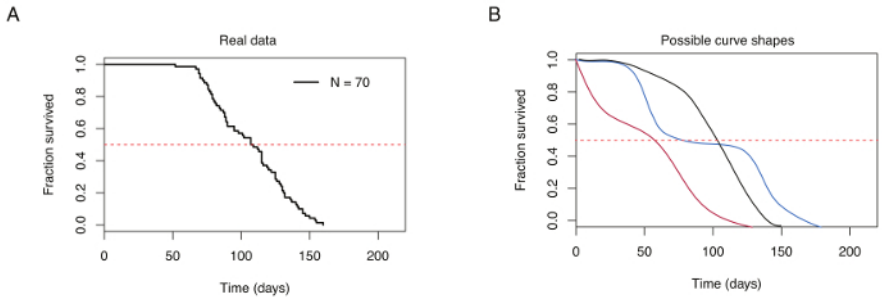

Figure 4: Representative survival curve for 70 male turquoise killifish. (A) Typical survival curve for laboratory-raised turquoise killifish. **(B)** Comparison of survival curves obtained from fish raised under optimal husbandry conditions (black) and poor husbandry (red and blue). The dashed red horizontal line indicates 50% survival, intersecting survival curve at median life span (indicated on the x-axis). Please click here to view a larger version of this figure.

ID	Forward primer	Reverse primer	Size range (bp)
***NfuSU0007**	GGCTAAGCCTTGCTGACAGA	CAGGGAGCTGAAAACCTCAG	166 - 214
***NfuSU0010**	CGCAGTCTGATCAAATCGTGT	TGTTTGAAGGTTCACATTCATTATC	220 - 272
NfuSU0016	CATGGCTAAACCGTGATGAA	GAAGGACGCCAGCTATGAAG	209 - 240
NfuSU0022	AACACAGCTCTCGTAAGGAGGTA	TTCAGACTTGTCTTACTACCATGTTT	198 - 238
NfuSU0027	TCCAGCTGAATCGGTAATGA	AAACTCGAGGGTGCAATCTG	164 - 226
NfuSU0049	CTGGACAAAGTGCCAATCAC	CTCCCACAGTCCCAAAACAT	196 - 197
NfuSU0050	CCAGAATGAACAATACTCAGATCAA	GCAGCTTAGTTTAATGATATCACAATG	252 - 295
NfuSU0060	CTAGCCACTCCCCTGGTTTA	CCGTCACGATGTGCTGATAC	216 - 248
NfuFLI0030	CAGAAGCTAAAGGCCAGACG	GGGAAACAATAGGGAACCAC	174 - 205
***NfuFLI0091**	ACGCTGACTCTACCCAGTC	CTGCCTGCTACTGACAATG	355 - 373
*- sex determination markers			

Table 1: Genotyping primers for strain identification.

Discussion

We describe a protocol for laboratory culturing of turquoise killifish, including embryo collection, incubation, as well as adult fish housing, breeding, and feeding. Our protocol is specifically targeted to laboratories that conduct research focused on adult fish, in particular for experimental studies on aging and life span. Turquoise killifish can be raised on a standard zebrafish facility; however, important aspects of killifish husbandry differ from standard zebrafish care[16]. These adjustments include early transition from a brine-shrimp only diet to a diet supplemented with protein-rich blood worm, as well as specific steps in embryo incubation, consisting of a liquid and dry incubation stage.

Critical steps within the protocol include shipping embryos within 8 - 30 °C temperature range. In case of breeding, fecundity depends on feeding frequency and food quality; therefore, we recommend at least two feedings a day per breeding tank to raise embryos yield (see section 5.6.). During embryo bleaching, do not extend embryo incubation in the bleaching solution. This may cause damage to the egg chorion and increased embryo mortality. When incubating embryos with methylene blue, do not prolong incubation of ready-to-hatch embryos for longer than 2 weeks as their viability will be dramatically reduced. For hatching turquoise killifish, low temperature of humic acid solution improves hatching and complete immersion of the embryos in the solution allows synchronized hatching. Not sufficient aeration during the incubation results in high rates of fry not able to fill the gas bladder ("belly-slider" phenotype, see Notes in section 5.1).

Limitation of the protocol for breeding includes the use of the sand substrate which poses challenges to centralized filtration systems and should be replaced by alternative methods in the future. Possible alternatives could be the use of zebrafish breeding tanks. Embryo bleaching could cause major physical-chemical changes in the egg chorion that could result in altered chorion physiology and hatching success. Constant exposure of embryos to methylene blue may induce long-term changes in adult fish physiology. Raising adult fish in individual tanks for survival cohort studies may negatively affect fish behavior and health. However, group housing for survival cohort studies adds significant confounding factors due to the establishment of social dominance and male territories, leading to strict social hierarchies. Therefore, we judge that isolation of male fish for survival studies is a reasonable compromise. Feeding laboratory killifish colonies with live food from un-controlled sources add a risk for external contaminations from parasites and potentially pathogenic microbial communities. In the future, an ad hoc sterile fish feed should be developed.

Future improvements to this protocol will focus on a controlled, non-live diet, which still leads to completing sexual maturation within 3 - 4 weeks. In summary, our protocol offers accessibility to turquoise killifish laboratory culturing to a wide scientific community.

Disclosures

All the authors declare no competing financial and non-financial interests.

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References

1. Valenzano, D. R., Aboobaker, A., Seluanov, A., & Gorbunova, V. Non-canonical aging model systems and why we need them. *EMBO J.* **36** (8), 959-963 (2017).
2. Harel, I., & Brunet, A. The African Turquoise Killifish: A Model for Exploring Vertebrate Aging and Diseases in the Fast Lane. *Cold Spring Harb Symp Quant Biol.* **80** 275-279 (2015).
3. Smith, P. *et al.* Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. *Elife.* **6** (2017).

4. Cellerino, A., Valenzano, D. R., & Reichard, M. From the bush to the bench: the annual Nothobranchius fishes as a new model system in biology. *Biol Rev Camb Philos Soc.* **91** (2), 511-533 (2016).
5. Kim, Y., Nam, H. G., & Valenzano, D. R. The short-lived African turquoise killifish: an emerging experimental model for ageing. *Dis Model Mech.* **9** (2), 115-129 (2016).
6. Blazek, R. *et al.* Repeated intraspecific divergence in life span and aging of African annual fishes along an aridity gradient. *Evolution.* **71** (2), 386-402 (2017).
7. Terzibasi, E. *et al.* Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. *PLoS One.* **3** (12), e3866 (2008).
8. Kirschner, J. *et al.* Mapping of quantitative trait loci controlling lifespan in the short-lived fish Nothobranchius furzeri--a new vertebrate model for age research. *Aging Cell.* **11** (2), 252-261 (2012).
9. Valenzano, D. R. *et al.* Mapping loci associated with tail color and sex determination in the short-lived fish Nothobranchius furzeri. *Genetics.* **183** (4), 1385-1395 (2009).
10. Reichwald, K. *et al.* Insights into Sex Chromosome Evolution and Aging from the Genome of a Short-Lived Fish. *Cell.* **163** (6), 1527-1538 (2015).
11. Valenzano, D. R. *et al.* The African Turquoise Killifish Genome Provides Insights into Evolution and Genetic Architecture of Lifespan. *Cell.* **163** (6), 1539-1554 (2015).
12. Harel, I. *et al.* A platform for rapid exploration of aging and diseases in a naturally short-lived vertebrate. *Cell.* **160** (5), 1013-1026 (2015).
13. Valenzano, D. R., Sharp, S., & Brunet, A. Transposon-Mediated Transgenesis in the Short-Lived African Killifish Nothobranchius furzeri, a Vertebrate Model for Aging. *G3 (Bethesda).* **1** (7), 531-538 (2011).
14. Harel, I., Valenzano, D. R., & Brunet, A. Efficient genome engineering approaches for the short-lived African turquoise killifish. *Nat Protoc.* **11** (10), 2010-2028 (2016).
15. Polacik, M., Blazek, R., & Reichard, M. Laboratory breeding of the short-lived annual killifish Nothobranchius furzeri. *Nat Protoc.* **11** (8), 1396-1413 (2016).
16. Avdesh, A. *et al.* Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. *J Vis Exp.* (69), e4196 (2012).