STANDARDIZED LABORATORY PROTOCOL FOR HOUSING OF *NOTHOBRANCHIUS FURZERI*

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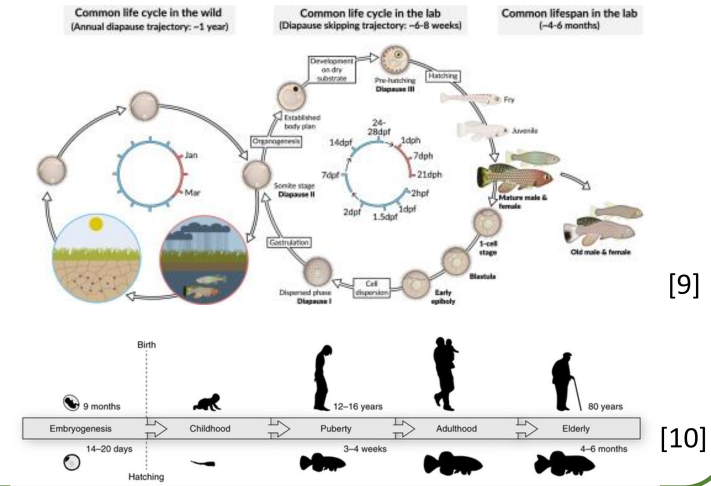
ZENOLAB

Objectives

Nothobranchius is a genus of the family Cyprinodontiformes, that comprises 43 described and several undescribed species. *Nothobranchius* range from native of Eastern and Southeastern Africa, an area of distinct dry and rainy seasons. The annual fish turquoise killifish *Nothobranchius furzeri* is the shortest-lived vertebrate which can be cultured in captivity and its short median lifespan reflects an adaptation to the ephemeral nature of the habitat. During the dry season, the ponds can desiccate and the population survives as drought-tolerant embryos. When the ponds are filled with water, the embryos hatch, rapidly grow and reach sexual maturation after few weeks [1]. Thanks to the short lifespan, the rapid growth, the early sexual maturation and the possibility to rapidly assess the effects of genetic and nongenetic interventions on aging and aging-associated phenotypes, *N. furzeri* is becoming an increasingly popular model organism for aging research [2]. The current turquoise killifish laboratory strains include: 1) GRZ, an inbred strain, that has a median lifespan of 9–11.5 weeks and 2) MZM-04/03 and MZM-04/10, that have a median lifespan of more than 21–28 weeks [3].

Although the killifish has gaining popularity in the scientific community as an important model system for the study of vertebrate aging [4, 5], husbandry and housing protocols already published [6, 7, 8] are often not affordable and do not provide repeatable conditions. In fact, rapid growth, maturation and aging make maintenance in captivity of *N. furzeri* laboratory strains challenging. With this work we want to establish the best and simplest husbandry and housing protocol in standardized laboratory conditions.



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Methodology

EMBRYO HUSBANDRY

EMBRYO COLLECTION

- Collect eggs from the breeding tank and transfer them to a 90 mm Petri dish in ~40 mL of autoclaved system water.
- Inspect embryos in 90 mm Petri dish under a light stereomicroscope and remove all the damaged and not fertilized one.

EMBRYO BLEACHING

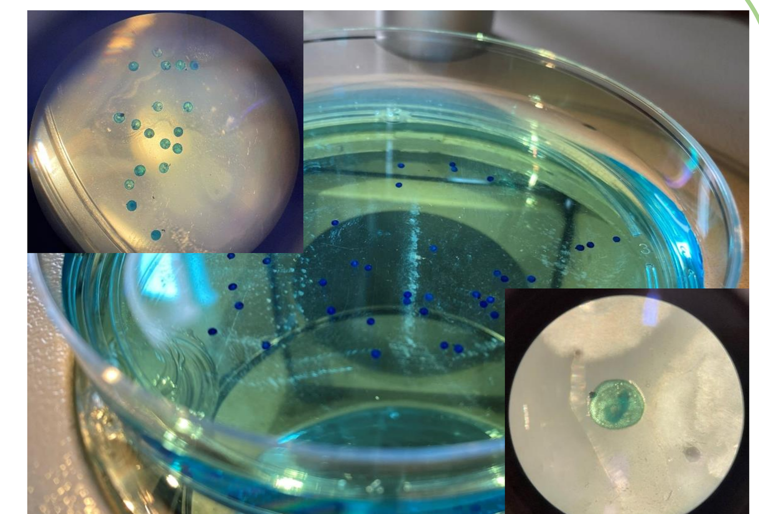
- Remove the system water from the Petri dish and add 50 mL of freshly prepared H2O2 (1% v/v in autoclaved system water).
- Shake embryos for 5 min.
- Remove H2O2 solution and wash embryos three times for 5 min with 50 mL of methylene blue solution (2ml of 0.1% methylene blue to 1 liter of autoclaved system water).
- Remove methylene blue solution and add 50 mL of H2O2 (1% v/v in autoclaved system water) to embryos and shake for 5 min.
- Remove H2O2 solution and wash three times for 5 min with 50 mL methylene blue solution.
- Incubate embryos at 28 °C at 12h light/12h dark to increase synchronous embryos development, at a maximum density of 50 embryos per 90 mm Petri dish in 40 mL of methylene blue solution.

EMBRYO INCUBATION IN METHYLENE BLUE

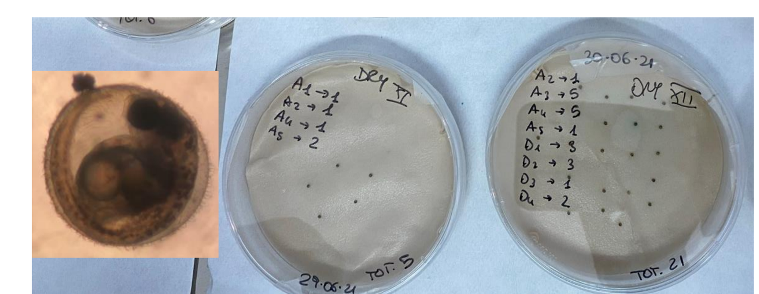
- Daily inspect incubated embryos, removing any dead embryos and replace with fresh methylene blue.

EMBRYO TRANSFER TO FILTER PAPER

- As developed embryos will have visible and complete black eyes transfer embryos from the methylene blue solution onto a previously prepared filter paper plate.
- Filter paper plates are 90-mm Petri dish where 3 layers of filter paper disks are placed inside. Add 5 mL of humic acid solution to keep humidity.
- Humic acid is prepared by dissolving 1 g/L humic acid (53680-50G Sigma Aldrich) in system water. Autoclave and store at 4 °C for 2 weeks.
- Spread embryos ~5 mm apart with forceps, up to 40 embryos per 90 mm plate.
- Seal the Petri dish with parafilm.
- Incubate embryos at 28 °C at 12h light/12h dark . Once a week the plates are opened to check embryos and humidity. If necessary add humic acid, never directly on the embryos.
- When embryos have fully developed golden irises and the corion appear thinner and more transparent, they are ready for hatching.



Embryos in methilene blue

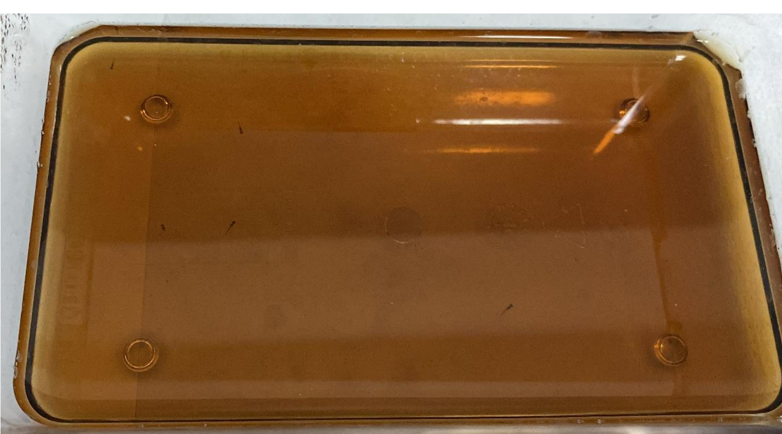


Embryo with full black eyes and filter paper plates

HATCHING

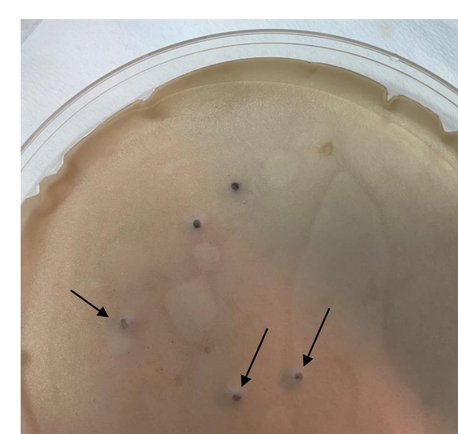
- Prepare the hatching box on the work bench: hatching box must be cold from freezer, fill humic acid fresh from the fridge, tilt the box and add an air supply.
- Transfer carefully up to 20 developed embryos into the hatching box. Make sure that all embryos are completely immersed. The humic acid solution must be shallow, not deeper than 2 cm.
- After four hours, remove air supply, level the hatching box, replace humic acid with fresh one and leave it like this until the next morning.
- Transfer unhatched embryos back to the solid substrate. One of the most frequent problems is plate hatching: retry hatching after three days.
- Transfer hatched embryos into a new tank.
- Upon hatching, turquoise killifish are readily capable to uptake and consume live food. For optimal growth, feed three times per day with excess freshly hatched brine shrimp (*Artemia salina*).
- From the day after hatching add in the tank autoclaved system water once a day in the proportion of 1:1 and dissolve ¼ of an oxygen tablet every three days.

Embryo with golden irises and hatching box



Hatched embryos

Hatching problem: part of the corion persists around the head of the embryo



Hatching problem: arrows indicate plate-hatched embryos that have developed molds

Set up for culturing brine shrimp and larvae with orange-colored abdomens, sign of full satiation

RAISING JUVENILE AND ADULT FISH

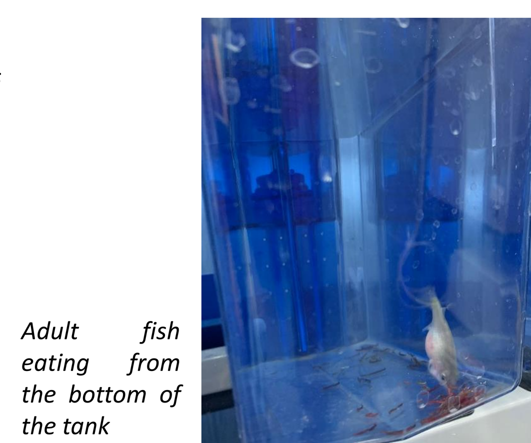
- Feed juveniles three times per day with freshly hatched brine shrimp in excess until 21 days post-hatching. Siphon out debris from the bottom of each tank daily.
- At 21 days start weaning by feeding the juvenile once with the bloodworms and twice with brine shrimp. We used frozen *Chironomus spp*.
- At 28 days transfer juvenile fish to 3.5 L tank equipped with a 300 µm baffle into the re-circulation system.
- At 4 weeks start adult feeding: fish are fed three times a day with 400mg/day/fish of bloodworms. The fish are able to eat even from the bottom of the tank.
- At this stage ensure that fish reach complete sexual maturation. Females can be co-housed at a density of up to 4 females per 3.5 L tank while males must be housed alone. It is possible also to create a group with one male and two females.

BREEDING

- Mating involves one male and two females.
- Classical breeding tank for turquoise killifish is composed by a plastic container (10 x 10 x 5 cm) filled with autoclaved sand reaching a final depth of ~2 - 3 cm that is placed in the center of the breeding tank.
- Possible alternatives could be the use of zebrafish breeding tanks.
- Let turquoise killifish breed every 10-14 days.

Frozen chironomus spp

Weaning moments



Adult fish eating from the bottom of the tank

Examples of breeding tanks

Conclusions

Our purpose is to define a standard protocol for *Nothobranchius furzeri* management: standardization protocols provide ranges of husbandry parameters that promote greater reproducibility of experimental work. With this work we set up detailed notes for eggs incubation, hatching, daily care of juvenile and adult fish, feeding and breeding. We are now planning to develop a unified laboratory diet using exclusively dry pellets [11]. We trust that taken together these notes provide a basis for facility heads, husbandry personnel, veterinarians and other stakeholders to discuss further improvements in *N. furzeri* care.

Fundings

This research was funded by the grant of the University of Camerino, FAR 2019 (Rector's Decree 137/2020) "The African turquoise killifish *Nothobranchius furzeri*: a new model of a diet-induced obesity - KILLOB" and by Prin 2022 – 2022LYPBCT "Notho-Diet: from chemosensory perception to husbandry standardization".

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