Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*

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Turquoise killifish, *Nothobranchius furzeri*, have an intrinsically short life span, with a median life span of <6 months and a maximum (90%) life span of 9 months. This short life span, which is unique among vertebrates, evolved naturally and has resulted in *N. furzeri* becoming a widely used laboratory model species in aging research and other disciplines. Here, we describe a protocol for the maintenance and breeding of the species under laboratory conditions. We provide details for egg incubation, hatching, everyday care of juvenile and adult fish, breeding and treatment of most common diseases. Emphasis is given to the fact that the requirements of *N. furzeri* substantially differ from those of other fish model taxa; *N. furzeri* live brief lives and in nature undergo nonaquatic embryo development, with consequences for their laboratory culture.

INTRODUCTION

Fishes combine a vertebrate body plan and physiology with a relative ease of culture and high reproductive output, and several fishes have become proven model taxa. Traditional laboratory fish models such as zebrafish (*Danio rerio*), guppies (*Poecilia reticulata*) and medaka (*Oryzias latipes*) are widely used in biological research[1,2]. Others such as the fathead minnow (*Pimephales promelas*) and the stickleback (*Gasterosteus aculeatus*) have become fundamental models in particular disciplines[3,4]. However, in terms of one particular life history trait—the life span—traditional fish model species, as well as all other vertebrates, fall short compared with non-vertebrate animal models. Vertebrates simply live too long to be practical for many research agendas.

Among fish, medaka, guppies and zebrafish all live ~4–6 years[2,5,6]. By contrast, many annual killifish complete their entire life span in well under a year. Annual killifish live in temporary freshwater pools in Africa and South America, where their posthatching life span, typically only a few months, is limited by the seasonal desiccation of their habitat. This short life span is intrinsic—they live similarly short lives under laboratory conditions and undergo the same functional deteriorations associated with typical vertebrate aging[7]. Among the annual killifish, the shortest life span is exhibited by members of the African genus *Nothobranchius*, comprising ~70 species[8], with the shortest life span reported for *N. furzeri* Jubb, originating from a relatively arid region in the southern periphery of the *Nothobranchius* range.

Advantages of *N. furzeri* as a lab model

N. furzeri is the species possessing the shortest life span among vertebrates that can be cultured in the laboratory[9], and it has assumed the role of 'vertebrate fruit fly'[10]; the median posthatching life span of wild-derived laboratory-bred *N. furzeri* is <6 months[11,12], only twice that of *Drosophila melanogaster*[13]. Established inbred lines have even shorter life spans[9,11]. The longest-lived *N. furzeri* individual in our breeding facility reached 17 months, an observation corroborated by reports from other laboratories[14].

The short life span of *N. furzeri* is combined with other characteristics that facilitate its use in laboratory-based research. *N. furzeri* is a small fish (maximum length 7 cm; **Fig. 1**), and because of its origin in space-limited temporary pools it is very undemanding in terms of aquarium space. As a result of its subtropical natural distribution (southern Mozambican savanna), it tolerates water temperatures from 15 to 35 °C, including rapid fluctuations[15]. Behavioral observations, including those of mating behavior, are feasible because the fish are naturally bold and reproduce on a daily basis. A pair begins spawning within minutes (often seconds) after being paired, and the species is very prolific. Under optimum food rations, a single female produces 30–50 eggs each day. Embryonic development varies between 17 d and 3 years; this variability is the result of three facultative embryonic diapauses[16,17] (**Fig. 2**). Eggs are incubated in a damp environment (and can be easily shipped at this stage) and synchronized hatching of experimental cohorts can be achieved when the eggs are wetted. Upon hatching, maturity is reached in <3 weeks[18].

Methodological innovations in laboratory research on *N. furzeri* include the production of various inbred strains, including one fully inbred (homozygous) and perfectly viable strain[19]; existence of an annotated brain atlas[20]; description of histopathologies, including abundant occurrence of tumors[7]; successful transgenesis[21–23]; an annotated transcript catalog[24]; age-dependent

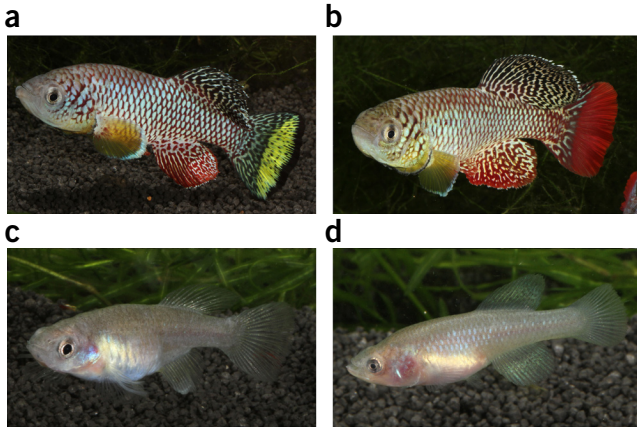

Figure 1 | Annual fish *Nothobranchius furzeri*. (**a**–**d**) Yellow form, male (**a**); red form, male (**b**); adult female (**c**); and maturing male (**d**). Note opalescent hue on flanks and black pigmentation in the dorsal fin in **d**, temporary colors preceding the appearance of red, yellow and blue colors. Dorsal and anal fin are also relatively larger than in a female.

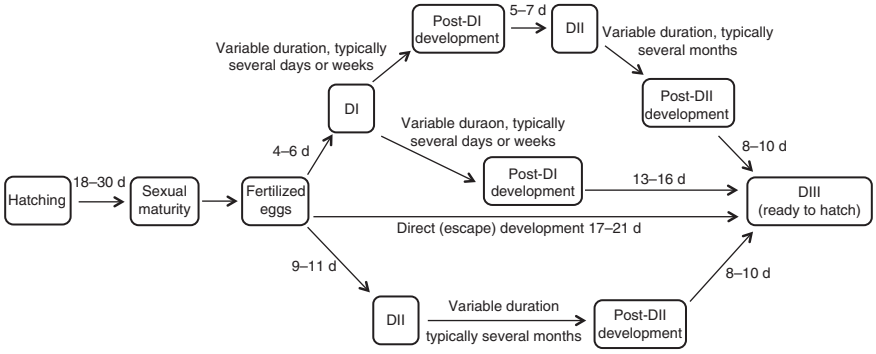

Figure 2 | Developmental trajectories of *N. furzeri* in aquatic medium (egg incubation at 25 °C). Note that the exact timing of developmental processes varies greatly with ambient temperature. DI, diapause I; DII, diapause II ; DIII, diapause III.

microRNA expression profiles[25]; an assembled genome[26,27]; and successful application of a genomic and genome-editing toolkit using CRISPR/Cas9 technology[28].

Another advantage is that the specific features that make *N. furzeri* such a valuable experimental animal have evolved naturally as adaptations to its environment[15,17,18,29–33], with natural trade-offs among life-history traits[34]. Temporary pools represent an unpredictable environment in which the prevailing principle is the ability to exploit chance events[34]. This also brings a caveat for laboratory work—*Nothobranchius* spp. have evolved very plastic responses to their environment[18] and a high degree of general 'sensitivity' to an environmental change. This feature extends into captive breeding and is manifested by their relatively broad reaction norms that generate phenotypic variability despite controlled laboratory conditions, especially in non-inbred lines. Although this might be viewed as an undesirable aspect of their life history for laboratory work, it also offers the opportunity to identify underlying regulatory mechanisms with potential application in human medicine[35].

Limitations of *N. furzeri* as a lab model

Current limitations of *N. furzeri* use for laboratory research include inconsistent estimates of life span and life history trait parameters among laboratories[9,11,12,14,18,36]. Given the strong influence of ambient temperature, food rations and housing conditions (fish density per volume of water, social versus individual housing) on the growth, reproduction and life span of *N. furzeri*, these inconsistent estimates are partially due to a lack of a commonly accepted and standardized protocol. A second limitation is the lack of artificial diet that would enable standardization of food quality among laboratories and across experiments within individual laboratories. Although a relatively satisfactory level of standardization can be achieved for juvenile food using several brands of widely available *Artemia* eggs, the quality and energy content of the adult diet—frozen and live bloodworm (*Chironomus* larvae)—are locally variable. No artificial diet is currently available.

Diseases of *N. furzeri*

In the wild, *Nothobranchius* spp. are typically infected by parasites with complex life cycles: survival of the parasites during the dry season and their transmission between temporary pools is enabled by definitive hosts such as birds. In captivity, *N. furzeri* commonly suffers from several diseases (**Fig. 3**). The risk of being infected with a pathogen is greatly increased by the use of live and frozen food from sources with no guarantee of a pathogen-free product. By far the most common disease is oodinosis (velvet), which is caused by a dinoflagellate, *Piscinoodinium pillulare*. *P. pillulare* infestation causes a sort of yellowish coating consisting of miniature dots on the fish's body and fins[37] (compare **Fig. 3a** with **Fig. 3b**). The fish can become infected through mutual contact or live food. It is also possible that the oodinosis infection is latent in captive *Nothobranchius* populations, shows no symptoms for long periods of time, with outbreaks triggered under deteriorated conditions (for diagnostics and treatment of oodinosis, see Step 25 of the PROCEDURE).

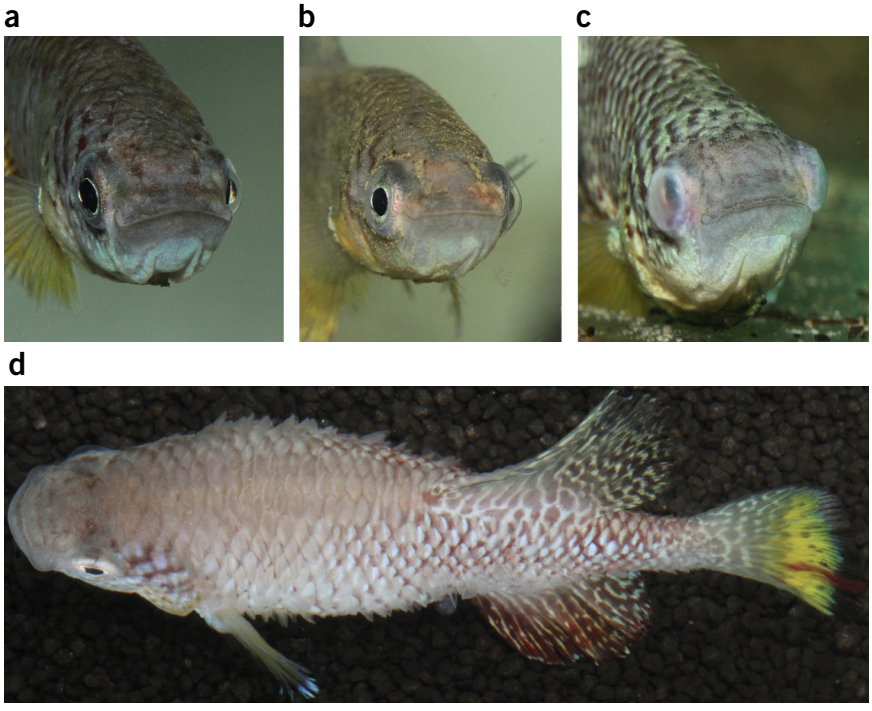

Figure 3 | External appearance of fish infected by common *N. furzeri* pathogens. (**a**–**d**) Healthy *N. furzeri* male (**a**); *N. furzeri* male infected with velvet (*P. pillulare)*—note the yellowish spots on the body (**b**); (**c**) *N. furzeri male* infected with unspecified bacterial infection (here manifested as protruding eyes—exophthalmos) and (**d**) *N. furzeri* male showing symptoms of dropsy.

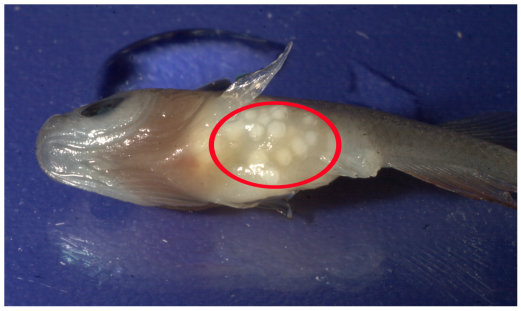

Figure 4 | Male *N. furzeri* infected with *Glugea* sp. Xenoparasitic complexes of *Glugea* sp. are visible as white cysts marked by a red ellipse. Photograph courtesy of Alexander Dorn.

N. furzeri fed unsound food may suffer from unspecified infections of supposedly bacterial origin. These manifest as a general decrease in activity and appetite, together with some morphological changes (for the diagnostics and treatment of unspecified infections, see Step 28 of the PROCEDURE), even when fish are under otherwise optimal care. Particular diseases such as dropsy (**Fig. 3d**) and *Glugea* are untreatable. The symptoms of dropsy are shared by several agents, including bacteria and viruses[37], and typically only a small number of individuals in the stock are affected (for diagnostics, see Step 26 of the PROCEDURE). Most of the agents are ubiquitous and cause a problem only when fish care is poor (e.g., insufficient water change). *Glugea* sp. is a unicellular microsporidian that forms 'xenomas' inside the host cells (**Fig. 4**), which causes parasitic growth[38] (for diagnostics, see Step 30 of the PROCEDURE). When it occurs, it has the potential to destroy entire stocks.

Experimental applications for *N. furzeri*

Some *Nothobranchius* species, especially *N. guentheri*, were used as experimental animals in laboratory studies in the 1970s and 1980s, including studies of early embryogenesis[39–41], diapause[16,42–44], histopathology associated with aging[45,46], toxicology[47] and behavior[48]. A renewed interest leading to intense scientific attention started only after the publication of a study that demonstrated the extremely short natural life span of *N. furzeri* in 2003 (ref. 9). This prompted the suggestion to use *N. furzeri* in aging research[49]. It was the first vertebrate model to demonstrate that resveratrol (a natural antioxidant) may extend life span and retard the expression of age-related markers[50]. Further studies showed that *N. furzeri* aging is also decelerated by lower ambient temperature[51] and dietary restriction[52]. Other applications that used laboratory breeding of *N. furzeri* include description of histopathologies including abundant occurrence of tumors[7], demonstration of aging-related telomere shortening despite high telomerase activity[53], aging-related impairment of mitochondrial function[54], age-dependent decline in adult neurogenesis[55], temporal pattern of microRNA expression[25], mapping of quantitative trait loci controlling life span[56] and regenerative capacity[14,57]. Comparative studies of aging were also performed among *N. furzeri* lab strains[11] and among *N. furzeri* and related species[12,58].

Laboratory breeding of *N. furzeri* was further used in studies of developmental bet-hedging in *N. furzeri* embryonic development and diapause[17], which led to within-population alternative life-history strategies[34]. *N. furzeri* has great potential in research into ecology and evolution[19]; studies on resource allocation and compensatory growth[59] and on reproductive isolation among sympatric species[60] have been completed, and several other studies are in progress. Notably, the short generation time makes *N. furzeri* an apposite model for experimental evolution studies.

TABLE 1 | Comparison of key aspects of husbandry between *N. furzeri*, zebrafish *D. rerio* and medaka *O. latipes*.

Aspect of husbandry	*N. furzeri*[11,12]	*D. rerio*[64,65]	*O. latipes*[66–69]
Embryonic development	Erratic	Predictable	Predictable
Hatching success	Variable	Stable	Stable
Onset of feeding after hatch	Immediate	Delayed	Delayed
Raising juveniles on an artificial diet	Currently unavailable	Available but suboptimal	Currently unavailable
Adult artificial diet	Currently unavailable	Available	Available
Food quantity requirements	High	Normal	Normal
Age at maturity	3–4 weeks	12–16 weeks	6–12 weeks
Spawning periodicity	Multiple events each day (30–50 eggs daily)	Potentially daily, typically once in a couple of days (100 eggs per spawning)	Daily (1–14 eggs)
Specific spawning substrate	Not required	Not required	Required
Intraspecific aggression	Male aggression—serious risk of injury	Negligible risk of injury	Low risk of injury
Maximum laboratory life span	1.5 years	>5 years	4.5 years
Median laboratory life span	0.5 year	3.5 years	2 years

Overview of the procedure

This *N. furzeri* laboratory husbandry protocol is based on 10 years of first-hand experience in breeding *Nothobranchius* spp. for scientific purposes. All procedures and methods presented herein have consistently proven to be effective[18,34,59]. The protocol emphasizes common laboratory practices and is intended to be more compact than the manual by T. Genade (available at http://www.nothobranchius.info/pdfs/lab_protocols_1.pdf). We have found some of the advice therein ineffective (e.g., sinking the eggs in a deep body of water to promote hatching), unnecessary (e.g., transfer of the freshly hatched fish to a new container) or unnecessarily laborious and inapplicable in large-scale laboratory husbandry (e.g., the CO_2 exhalation hatching method). We have tried to make the current protocol applicable across laboratories in order to promote greater reproducibility of experimental work using *N. furzeri* as a model.

This protocol describes how to maintain and breed *N. furzeri* and is not intended to standardize particular experimental procedures such as estimates of survival or aging-associated declines across laboratories. In the PROCEDURE section, we provide details for hatching, raising and breeding *N. furzeri*, as well as incubating its eggs and curing its most common diseases.

The key factor for successful culturing of *Nothobranchius* is to recognize and accept their difference from other small laboratory fishes (**Table 1**). As we have imported a number of *N. furzeri* populations from the wild, we often receive requests for *N. furzeri* eggs from other laboratories. Of these, many initial attempts to establish a breeding colony fail for reasons that have not been determined. We believe this often stems from a lack of appreciation of the important respects in which the culture of *N. furzeri* differs from that of zebrafish or guppies. *N. furzeri* grow and live very fast; they need greater food rations, more nutritious food and frequent water exchange to remove metabolic waste products. Their embryonic development is often asynchronous (**Fig. 2**), influenced by the incubation conditions (**Fig. 5**), which results in substantial variability in actual hatching rate.

For those seriously interested in long-term research using *N. furzeri*, we recommend gaining initial experience with species that are easier to breed, such as *Nothobranchius korthausae* Meinken and *Nothobranchius guentheri* Pfeffer. When keeping *N. furzeri*, we recommend producing a large number of eggs to mitigate any potential problems with embryo development, hatching and raising the fish. Eggs can be easily obtained in large quantities when parental fish are fed large amounts of high-quality food[18,59]. Most initial problems can be overcome with a sufficient egg reserve and rapid production of a new generation of fish.

Experimental design

The protocol can be modified to effectively use locally available resources. Given that the expected outcome is the maintenance of *N. furzeri* and production of experimental animals rather than direct experimental assays, such modification should be straightforward to implement. However, caution should be taken, and any substantial departure from this protocol should be clearly stated. Notably, transgenerational effects (maternal or epigenetic) have been reported in annual fishes[61], and therefore as a precaution the researcher should assume that the social and nutritional state of parental fish can be translated into the phenotypic expression of their offspring, with potential consequences for analysis of functional traits.

Embryo incubation. We provide three basic alternatives for embryo incubation—in aqueous medium, on top of damp peat and inside of damp peat. The methods vary in their effect on embryo mortality rate[18]; each is useful under certain circumstances and each predisposes embryos to a different pace of development, with important consequences for hatching success and posthatching performance (see Step 16 of the PROCEDURE and **Fig. 5** for details). Embryos that develop directly (without diapause) tend to live faster and shorter lives than embryos that

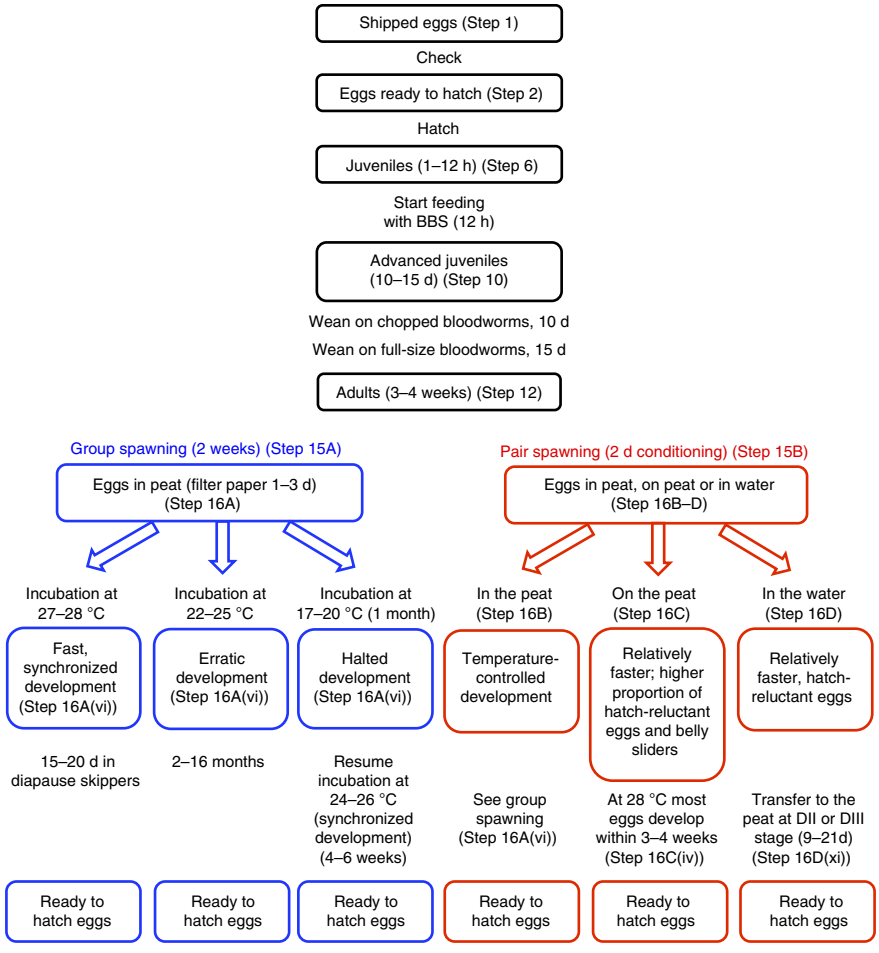

Figure 5 | Schematic overview of the sequence and duration of different steps in the husbandry regime of *N. furzeri*. BBS, baby brine shrimp.

are developed via diapause[34]. We urge that the embryo developmental method be stated in the Methods of research papers.

Water quality. In the wild, *N. furzeri* inhabit bodies of water that vary widely in quality, including water chemistry, temperature, and depth as well as the frequency of fluctuations of these parameters[15]. In captivity, we recommend the use of hard water (high concentration of calcium and magnesium salts) with alkaline pH. Soft water (with lower conductivity) is tolerated by *N. furzeri*, but it has a lower buffering capacity and the fish are subsequently more prone to particular infections (e.g., Step 25 of the PROCEDURE). We recommend maintaining water temperature at 28 °C to enhance fish growth rate and egg production. However, temperatures ranging from 24 to 32 °C are readily tolerated by *N. furzeri*. It is important to keep in mind that metabolic rate scales with ambient temperature, with higher food requirements (and faster waste product accumulation) at higher ambient temperature (and vice versa).

Feeding. Feeding has an important effect on the growth and fecundity of *N. furzeri*. *Ad libitum* feeding is the preferred approach for *N. furzeri* welfare and standardization. Despite ongoing efforts, an artificial diet for *N. furzeri* is not currently available, which leaves frozen and live food as the only possibilities. As far as we are aware, only a single product (Hikari Bio-Pure bloodworms) is artificially cultured in a supposedly controlled and infection-free environment, although its availability varies by country and the cost is relatively high. While its use in major experimental assays is recommended, it is not necessary for *N. furzeri* maintenance and breeding.

Housing conditions. Fish housing conditions and density affect individual growth rates. Social interactions decrease the growth rate of subordinate individuals, either through direct encounters or via chemical communication. However, for regular husbandry, a set of aquaria without a recirculation system is sufficient. For experimental purposes, a range of professional zebrafish racks can be used. We have experience with the FishBox system (AquaMedic). This system is relatively inexpensive but suffers from frequent malfunctions, and we cannot recommend it for wider use. When fish are housed individually, differences among individual growth rates are lower, although not absent. It must be emphasized that individual housing requires frequent movement of fish for spawning—female fish need to spawn ovulated eggs to retain reproductive capacity, and the failure to lay eggs may be fatal for female fish[34]. Spawning twice per week is recommended. The extra handling required diminishes most benefits of individual housing.

MATERIALS

REAGENTS

- Chlorine-free fresh water
- Peat: a peat mixture of pH 6.5 for growing orchids (Agro CS, cat. no. EAN 859 400 500 7192; see Reagent Setup for further information)
- Glass beads (substrate for pair spawning) with a diameter up to 0.5 mm (e.g., 0.25–0.5-mm glass beads; P-Lab, cat. no. R155361)
- *Artemia salina* eggs (baby brine shrimp (BBS)) juvenile food. Sanders Premium Artemia (Sanders Brine Shrimp)
- Frozen regular-size bloodworms (*Chironomus* spp.), not 'jumbo' bloodworms, (cat. no. 30240)
- Frozen Tubifex worms (*Tubifex* spp.; cat. no. 32920)
- Frozen fairy shrimp (*Artemia* spp., cat. no. 32464 or *Daphnia* spp.)
- Frozen zooplankton (Hikari; cat. no. 30320)
- Live bloodworms
- Live fairy shrimp
- Live glass worms (*Chaoborus* spp.)
- Live mosquito larvae
- Live zooplankton of local choice
- Dry food (supplementary food item). Animal-protein-based dried pelleted or granulated fish food (e.g., Exot Hobby, SAK 55 and SAK Energy)
- OxyTabs (JBL, cat. no. 20080)
- EasyStrips (chlorine test; Tetra, cat. no. 19544-00)
- NaCl solutions (4, 8–10, 100 and 360 g/l solutions; P-Lab, cat. no. R 39573)
- Dry sea almond *Terminalia cattapa* leaves (e.g., IT Philippines)
- Methylene blue (0.002 g/l solution; P-Lab, cat. no. R15141)
- 5% sodium hypochlorite solution (household bleach; Savo)
  ! CAUTION Bleach is a strong corrosive, and it releases toxic chlorine when in contact with acids. Avoid contact with eyes, skin and clothing. Wear protective gloves during handling.
- Sodium thiosulfate (in 5 g/l solution; P-Lab, cat. no. T 05302)
- Gentamicin sulfate (in 10 mg/l solution; P-Lab, cat. no. D.02-012E)
- Chlorine test strips (Tetra, cat. no. 19542-00)
- *N. furzeri* killifish. The fish can be obtained from an established laboratory stock, a specialized breeder or imported from the wild. The drought-resistant eggs of annual killifish are suitable for shipping by post **! CAUTION** Any experiments involving live fish must comply with relevant institutional and national regulations. Our husbandry facility has been certified by the Ministry of Agriculture of the Czech Republic (43245/2008-10001 and 43245/2008-17210). The procedures were approved by Academy of Sciences of the Czech Republic (125/2010, 138/2010 and 1/2016). All authors hold certificates for working with experimental animals issued by the Ministry of Agriculture of the Czech Republic (M.P., CZ 01283; R.B., CZ 01265; and M.R., CZ 01285).

EQUIPMENT

- Glass aquaria, 6–80 l
- Free-standing air-driven sponge filters, e.g., model XY 2837, 16 × 15 cm (l × h) (Xinyou; **Fig. 6**)
- 4-mm-Diameter air tube (JBL, cat. no. 6108500)
- 12-mm-Diameter aquarium tubing (JBL, cat. no. 6108200)
- Hand nets (0.15–3 mm mesh)
- Thermostat-equipped submersible aquarium heaters (25–300 W; JBL, cat. no. 6042100)
- Plastic tubs (containers with lids, volume: 2 l; **Fig. 6**)
- Scalpel or box knife
- Glass jars for group spawning (volume 0.6 l, minimum 8-cm-wide opening; **Fig. 6**)
- Plastic zipper bags (5 × 8 cm for shipping eggs, 15 × 20 cm for egg incubation)
- Parafilm (P-Lab, cat. no. P701605)
- Fine entomological tweezers (soft grade, 0.2-mm steel thickness; Ento Sphinx, cat. no. 21.33)
- Standard dining spoon and teaspoon
- Compartmented (10 × 10 cm, 5 × 5 cells; P-Lab, cat. no. S000103; **Fig. 6**) and round (diameter 10–20 cm) Petri dishes
- Sieve with 1 mm mesh (e.g., a tea strainer; **Fig. 6**)
- Fine Tip Pasteur Pipettes (cut off tip to open to 3–4 mm diameter; Alpha Laboratories, cat. no. LW 4060; **Fig. 6**)
- Glass 'pipe' for manipulating fish (4 cm in diameter; Sklorex, cat. no. 531–2; **Fig. 6**)
- Laboratory wash bottles (0.5 liter; P-Lab, cat. no. K001638.N; **Fig. 6**)
- White cellulose filter paper (40 × 40 cm; Merci, cat. no. 480 622 080 040)
- Cooled laboratory incubator (e.g., Peltier cooled incubator; Thermo Scientific, cat. no. 3915FL)
- Recirculating system for individual housing of fish (e.g., FishBox; AquaMedic, cat. no. 403.10)
- Timer

REAGENT SETUP

Chlorine-free fresh water Use hard (15–18 degrees of general hardness, 450–550 μS/m^2, 7.0–7.5 pH) water for all applications in the protocol.

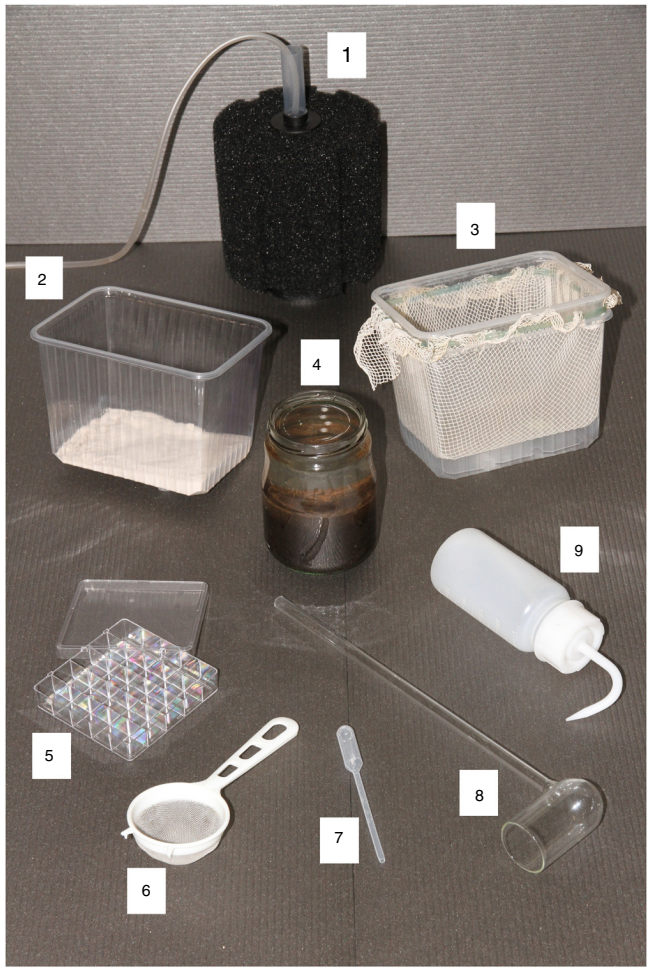

Figure 6 | Basic equipment needed for husbandry of *N. furzeri*. (1) Free-standing air-driven sponge filter, (2) spawning tub with fine glass beads, (3) spawning tub with mesh, (4) spawning jar filled with peat, (5) plastic incubation Petri dish with 25 compartments, (6) sieve (tea strainer) with 1 mm mesh, (7) disposable Pasteur pipette, (8) glass pipe for catching and transferring juvenile fish, and (9) laboratory wash bottle.

Soft water (50–300 μS/m^2) can also be used, but fish may be more prone to certain infections (see Step 25 of the PROCEDURE) and soft water has a lower buffering capacity against potential acidification by peat.

Sodium hypochlorite solution 5% Sodium hypochlorite solution (household bleach should be diluted to 1 ml/0.2 l water and 10 ml/100 l water).

! CAUTION Bleach is a strong corrosive and it releases toxic chlorine when in contact with acids. Avoid contact with eyes, skin and clothing. Wear protective gloves during handling.

NaCl solutions Mix 4, 8-10, 100 and 360 g of NaCl each with 1 l water.

Methylene blue solution Mix 0.002 g of methylene blue with 1 l of water.

Sodium thiosulfate solution Mix 5 g of sodium thiosulfate with 1 l of water.

Gentamicin sulfate Mix 1 ml of gentamicin sulfate with 999 ml of water.

Peat This is a highly locally diversified commodity—we are not aware of any suitable brand available worldwide. Peat with higher pH (>5) is more suitable. There are many peat-based gardening substrates intended for specific plants; some have higher pH and are suitable for spawning and ncubation. Ensure that the substrate contains no added fertilizers or hard particles such as pieces of wood or gravel (use a sieve to remove hard particles if necessary). Boil and rinse the peat repeatedly (2–3 times) to lower its acidity before use and to make it sink rapidly by removing trapped air.

***A. salina* eggs** Mix 1 liter of water, 8–10 g (one teaspoonful) of kitchen salt and an amount of BBS eggs depending on the number of fish to feed (assume three feeding events) in a bottle. Use 3 g of eggs to produce an amount of nauplii sufficient to feed at least 1,000 freshly hatched *N. furzeri* three times (one teaspoonful is 2.9–3.2 g). Note that hatching success varies with the brand of commercially available *Artemia* eggs and storage conditions; hatching success is 90% with Sanders Premium Artemia (Sanders Brine Shrimp), used in our lab. Adjust the egg quantity to local conditions if needed. Introduce strong aeration with airflow at the bottom of the bottle (an air stone helps to hold it there). Incubate the mixture at 23–28 °C. Collect BBS between 24 and 36 h after the start of incubation. Remove the aeration tube and allow hatched BBS to aggregate at the bottom of the bottle. Empty shells will float to the surface (~4 min). Siphon out BBS into a separate tub using a plastic (air) tube (diameter 4–5 mm). Use a Pasteur pipette (eye dropper) to transfer BBS to the fish aquarium; remnants of the salt solution do not pose a problem. Freshly hatched *N. furzeri* are capable of eating the entire size range of commercially available nauplii. **▲ CRITICAL** Hatched nauplii must be available at the time of fish hatching. Start incubating *Artemia* eggs at least 24 h before the fish hatching is planned.

EQUIPMENT SETUP

Aquarium setup for juvenile fish House juvenile fish (from the age of 3–4 d, see Step 8 of the PROCEDURE) in a tank with a minimum volume of 2 l per fish. Equip the tank with an air-driven, free-standing, removable filter with upward pointing outflow (**Fig. 6**). Keep the filter running in the tank for at least 3 weeks before adding fish, so that it is already colonized by bacteria. Keep the bottom of the aquarium bare; provide no shelters. Ensure that the filter outflow is not so strong that the current moves the fish involuntarily.

Aquarium setup for adult fish House adult fish in a tank with a minimum volume of 4 l per fish. Use the same tank setup as for juvenile fish. Provide only dim lighting to limit male–male aggression. For example, use a standard (60 W), center-positioned light bulb for a 3 × 4 m room with aquaria positioned near the walls. Do not use strong lighting directly above the aquaria, except when you are performing maintenance or checking the health and condition of the fish. Cover tanks with a lid to prevent fish jumping into neighboring aquaria (*N. furzeri* rarely jump but may do so when frightened). The use of a plastic lid rather than a glass lid is recommended. Drill a 5-cm hole in the center of the plastic lid to allow for easy handling and convenient feeding. Drill a hole at the lid's edge for the filter air tube and heater cord; ensure that the hole is not large enough for a fish to get through.

PROCEDURE

Hatching (shipped) eggs ● TIMING 6–12 h

1| Check the condition of the eggs after transport. Open the package (eggs are normally shipped in a small amount of peat in a plastic bag) and pour the peat into a Petri dish. Use a directed light source (e.g., standard table lamp, 25–40 W) to inspect the eggs, which are >1 mm diameter and can be seen with the naked eye. Gently stir the peat to search for the eggs. Eggs will be translucent with no apparent structures inside (**Fig. 7a**), opaque with an advanced embryo (**Fig. 7b**,**c**), or white when dead (**Fig. 7d**).

▲ CRITICAL STEP Do not allow the eggs to remain uncovered by the peat and exposed to low room humidity for longer than 5 min, to avoid their desiccation.

2| Estimate the proportion of ready-to-hatch eggs in the batch by checking their developmental stage. If the volume of the peat is substantial (>150–200 ml), check only a representative sample of the batch (e.g., 20% of the peat volume), as

Figure 7 | Gross staging of *N. furzeri* eggs incubated in peat. (**a**–**d**) Diapaused *N. furzeri* egg lacking structures that are readily visible to the naked eye (**a**); advanced, postdiapause II embryo with black-pigmented eyes (**b**); egg with a fully developed, ready-to-hatch embryo with gold-pigmented eye iris (**c**); and dead, decaying egg (**d**). Scale bar, 1 mm.

looking for killifish eggs in the peat can be time-consuming. Most eggs that are not yet ready to hatch are clear (no eye apparent; **Fig. 7a**), or sometimes black-pigmented eyes (**Fig. 7b**) can be seen. When eggs are ready to hatch, embryos with conspicuous, gold-pigmented irises can be seen (**Fig. 7c**). Count the number of eggs in the respective categories and estimate the proportion of ready-to-hatch eggs. If enough (~50 eggs should be enough to obtain a breeding group of adult fish to maintain a stock) are ready to hatch, proceed to Step 3 of the PROCEDURE. If only a small subset of eggs are to be hatched (e.g., for a specific experimental purpose), use fine entomological tweezers to gently move the ready-to-hatch eggs to 50–100 ml of boiled peat (see Reagents section) and proceed to Step 3 (the presence of peat promotes hatching). When moving eggs with tweezers, it is safer to transfer them in a small amount of peat so as not to damage them with the tweezer tips.

▲ **CRITICAL STEP** Hatching success may vary greatly in *N. furzeri*. We suggest picking out approximately twice the number of ready-to-hatch eggs required for experimental purposes.

▲ **CRITICAL STEP** Ready-to-hatch *Nothobranchius* spp. embryos remain viable for only a limited time, as they are gradually consuming their energy reserves. The precise time interval depends on the incubation temperature. The yolk sac begins diminishing ~60 d after an embryo reaches its pre-hatching stage[16]. Do not continue to incubate eggs for longer than 2 months after they reach the prehatching stage.

3| Pour out the peat containing the eggs into a small (6 l) empty aquarium. Select the size of the aquarium according to the volume of peat—the peat layer on the bottom should be no deeper than 2 cm so that the freshly hatched fish can easily swim out and not get trapped in the peat. Use a larger aquarium—or split the peat across more aquaria—to maintain a depth of 2 cm or shallower.

4| Pour cold water (15–16 °C) into the aquarium. Keep the initial water level at 3 cm above the peat (at the deepest end; **Fig. 8**). Immediately after wetting, gently disturb any larger clumps of peat (using fingers) to free any trapped embryos. Stir any floating peat to release residual air that may be causing it to float (repeat several times as required over the next 3 d until the peat stops floating). Ensure that the bottom of the aquarium is sloped so that the water level is zero at one side of the aquarium (**Fig. 8**). Although it is unclear whether newborn killifish need to gulp air in order to fill their swim bladders[62], they show a strong drive to reach the surface.

▲ **CRITICAL STEP** Low water temperature stimulates hatching; however, the water should be allowed to naturally reach room temperature (25–27 °C). This normally takes 2–3 h.

▲ **CRITICAL STEP** Wet the eggs at midday to allow for oxygen treatment (Step 5 of the PROCEDURE) and first feeding (Step 7 of the PROCEDURE) in the evening.

5| Prepare oxygen tablets by breaking each of them into 5–10 smaller pieces. At the point when most eggs have already hatched (depending on the estimated number of eggs) or 5 h after the eggs were wetted, add one broken-up oxygen tablet per 1 liter of hatching water. Fish begin hatching within 0.5–3 h and perform vigorous 'jerks', with most fish hatching within the first 5 h. The increased oxygen concentration in the water helps to minimize the number of fish that fail to fill their swim bladder[62]. Breaking the tablet into pieces increases oxygen concentration at a single time point, which is more important than maintaining high oxygen concentration over time. Note that incubation lengths are variable[16,17] (**Figs. 2** and **5**), and therefore it is uncommon for all eggs in a batch to develop synchronously; a variable proportion of undeveloped eggs may still remain in the peat after other embryos have hatched. The wetting often triggers dormant embryos from diapause; these become ready to hatch in 5–15 d (at 25 °C).

▲ **CRITICAL STEP** Do not postpone the application of the oxygen tablets. Late application (>24 h after hatching) is largely ineffective.

? TROUBLESHOOTING

Raising juvenile fish ● TIMING 3–4 weeks

6| Keep newly hatched fish in their hatching aquarium together with the incubation peat for 3–4 d post hatching to avoid manipulation of still-fragile fish. Re-arrange the sloped tank to a normal, flat position ~24 h after hatching. If early manipulation is required for experimental purposes, use a glass pipe to catch the fish while keeping them in water (**Fig. 6**). Do not use any aeration or filtration, and maintain water temperature at 25–27 °C by placing the tank in a heated room or using a small (25 W) submersible aquarium heater. Continue stirring the floating peat 3–4 times daily so that it releases residual air and settles to the bottom of the aquarium. To maintain adequate water quality, add 100% of the original (wetting) volume of water each day (e.g., in a 6-l aquarium (25 × 16 × 16 cm), this means raising the water level by 3–4 cm daily). Gently pour water (e.g., using a small-diameter, slow-flowing air tube as a hose or pouring the water onto the water surface covered by a plastic bag loosely floating on the water surface) so that the substrate on the bottom is not overly disturbed and the fish are not buried. For the first 3–4 d, a 6-liter tank can safely house 200–300 juveniles. Peat in the aquarium maintains a slightly acidic environment, keeping waste nitrogenous substances in a safer, ammonium form (NH_4^+).

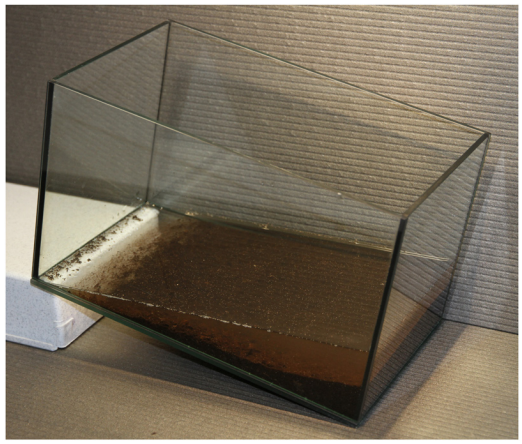

Figure 8 | Setup for hatching peat-incubated *N. furzeri* eggs. Sloped hatching aquarium with zero water level at one end and 3-cm water level at the other end.

7| For optimal growth, feed the young fish with *Artemia* nauplii (BBS) three times a day at regular intervals (morning, midday and late afternoon). Distribute the BBS evenly across the hatching aquarium. Ensure that all fish have full, yellow-colored bellies ~15 min after each feeding as a sign of full satiation. With an air tube, siphon out the substrate coating that emerges as a consequence of decayed BBS and that typically occurs at the brightest spot of the tank. The exact quantity of BBS to be used depends on the number of juveniles; initially, fish should be literally 'swimming in food,' but 1 h after feeding no more than 100–200 live nauplii should remain; these tend to aggregate at the brightest spot. The use of fatty-acid-enriched BBS (e.g., SELCO by Artemia Systems) is not required, as juvenile *N. furzeri* can achieve optimum growth rate on standard BBS[18].

▲ **CRITICAL STEP** If the eggs were overdue, feed fish as soon as possible—at the start of swimming, in conjunction with the oxygen tablet treatment. The appearance of the freshly hatched fish is a retrospective indication of the appropriate timing of the wetting. Overdue fish are very thin with no apparent yolk sac remnants.

8| At the age of 3–4 d, move the fish to a larger aquarium (see Equipment Setup). Slowly pour out all the water from the hatching aquarium to the new aquarium. Do not transfer the peat to the new aquarium (it will have sunk by now and remains at the bottom of the hatching tank). If you are in doubt whether all juvenile fish were transferred, refill the hatching aquarium with water from the new tank and wait until the peat resettles (the fine peat may make the water in the hatching aquarium very turbid when disturbed). Check for any remaining fish, and transfer them to the new tank using a glass pipe or by repeating the pouring procedure.

▲ **CRITICAL STEP** After the juveniles are moved to a larger tank, dry the peat from the hatching aquarium, as described in Step 16A of the PROCEDURE. Check the re-dried peat, as described in Step 2 of the PROCEDURE. If unhatched eggs are abundant, place the bag of eggs into an incubator (Step 16A of the PROCEDURE) and repeat wetting (Step 3 of the PROCEDURE) after 2–3 weeks.

9| Change 30% of the water (ensure that the drop in water temperature is <5 °C after the change) three times a week while siphoning out the waste from the bottom (use tubing with a 9-mm diameter). Continue feeding three times per day (juvenile *N. furzeri* should constantly have full, rounded bellies). For 5-d-old fish, two feedings per day is acceptable but suboptimal. To partly compensate for the decreased food delivery rate, add 16 g (a spoonful) of salt per 10 l of water; the increased salinity reduces osmotic shock and thus facilitates longer BBS survival in the aquarium, prolonging their availability to the fish.

10| At ~10 d of age, begin supplementing the BBS diet with frozen bloodworms at each feeding. Chop the bloodworms into smaller pieces for the first 4–5 d of bloodworm feeding, as full-sized bloodworms are still too large for the fish. Use a scalpel or a box knife to cut 2- to 4-mm-wide slices directly from a deeply frozen block. Melt the slices in a small amount of water and use a Pasteur pipette for feeding to the fish. Introduce the bloodworms first and wait for ~20 min before adding the BBS. Fish wean off the BBS and accept the new diet more easily when they are not distracted by other food items. Bloodworms are more nutritious than BBS, and therefore growth rates of *N. furzeri* have the potential to increase after weaning.

11| Assuming that the previous feeding regime and aquarium conditions were optimal, begin feeding full-size bloodworms (adult diet) at the age of 15 d. Separate any stunted individuals and place in another aquarium to avoid injuries from aggressive larger siblings during maturation. Some portion (5–10%) of the cohort may show stunted growth and be incapable of consuming full-size bloodworms at this age. This is an inherent interindividual variability rather than a sign of food shortage; it regularly occurs despite ideal feeding conditions. Continue the mixed BBS/chopped bloodworm diet with the separated individuals until they are capable of ingesting full-size bloodworms (1–7 d).
? TROUBLESHOOTING

12| Separate the most rapidly maturing male fish into a different aquarium to facilitate the growth and maturation of the other male and female fish (the rapidly maturing male fish can be returned to the same aquarium later). The first signs of secondary sexual characteristics appear at 12–25 d of age; sexual maturity can be reached at the age of 18 d (ref. 18). Initially, male fish are recognized by distinctly larger dorsal and anal fins, with signs of male coloration developing 1–3 d later (**Fig. 1d**). Mature female fish have bellies swollen with eggs (**Fig. 1c**) (from ~25 mm length). Most fish become sexually mature by 4 weeks of age under proper conditions.

Care of the adult fish ● TIMING 3–9 months

13| Keep adult fish density at a maximum of 4 l of water per fish, and use the same aquarium setup (see Equipment Setup) and water exchange regime as for advanced juveniles. Alternatively, house the fish individually in a recirculating system (**Box 1**). Maintain a temperature range of 27–32 °C. Prevent excessive harassment of female fish by maintaining equal or female-biased sex ratios in the aquarium (male fish relentlessly court female fish). Note that occasional nonlethal (or even lethal) male–male interactions do occur. Try to identify the particularly aggressive individual by direct observation (often the male with the least damaged fins) in case lethal interactions are repeated. Remove the aggressive individual(s) from the aquarium.
▲ **CRITICAL STEP** Beware of strain contamination in cases in which several *N. furzeri* strains are maintained in the same room. Keep all tanks clearly labeled with strain ID, date of birth and number of male and female fish. As a precaution, keep the tanks covered (see Equipment Setup) and try to avoid placing tanks with different strains adjacent to one another.

14| Feed fish twice per day (morning and evening) to obtain high reproductive output. Use frozen bloodworms as a staple diet—the fish consume bloodworms willingly while producing decent clutches of 20–40 eggs. For standardized experimental conditions, the exclusive use of frozen, cultured bloodworms (Hikari) is recommended. For breeding, feed the fish with live food from a pathogen-free source to maximize fish condition. Occasionally, supplement the diet with live and frozen brine shrimp, zooplankton or animal-protein-based fish pellets (e.g., SAK 55 or SAK Energy by Exot Hobby). When choosing an

Box 1 | Individual housing of *N. furzeri* in a recirculating system ● TIMING 3–9 months

For their entire life span, *N. furzeri* can be housed individually in small tanks in a recirculating system as an alternative to group housing. The advantages of individual housing include the following: (i) identification of individual fish; (ii) limiting the effects of social environment on growth rate, maturation and life span (independent replicates); (iii) limiting the risk of intraspecific aggression (only to spawning periods); and (iv) limiting the potential for spread of disease (provided that an UV lamp is used).

The disadvantages of individual housing include the following: (i) imposing differences from natural conditions; and (ii) increasing requirements for fish care, including the labor of egg collection and the need for more sophisticated equipment.

1. Prepare a recirculating system. Commercially available systems for zebrafish are generally suitable, provided that the volume of a single tank is at least 2 l and the water inflow is up to 0.6 l/min or water current is broken down by a screen.
2. Fill the system with water. Turn on the water pump and allow a bacterial community to develop in the filter by keeping it running for at least 3 weeks.
3. Measure the temperature of the water and use a powerful, thermostat-equipped heater (300 W) if necessary. Turn the UV lamp on.
4. Introduce the fish. Water filtration can be turned on when the fish reach the age of 10–15 d (the onset of bloodworm feeding; BBS would be filtered out). Use a screen (fry screen) to prevent the small fish being washed down the filter system.
5. Maintain a normal feeding regime (twice per day). Turn on the lighting during the feeding events to check the condition of fish but do not keep the lights on permanently. Change 30% of the water and siphon out organic debris accumulated on the bottom of the tanks twice per week (unless the recirculating system is designed for automatic waste collection). Clean filter media once every 3–4 weeks (follow the manufacturer's recommendations).
6. Collect the eggs from the fish using the pair-spawning method (Step 15B of the PROCEDURE) twice a week.

▲ **CRITICAL STEP** *N. furzeri* female fish continuously produce relatively large egg masses if they are fed well. If they are not allowed to spawn regularly, some individuals (30–80%) become egg bound (extremely swollen belly, eggs visible through the abdominal wall) and eventually die. Some female fish can release the eggs even without a male, but the initial capability is often lost within weeks to months.

alternative locally available brand, keep in mind that red or reddish food coloration is an important attractant. Note that dried food (pellets and flakes) cannot be the main component of *N. furzeri* diet, as the fish only reluctantly eat dried food. A rounded belly is a reliable sign of satiation. Siphon out any uneaten, nonlive food from the tank up to 2 h after feeding to prevent deterioration of water quality.

Breeding

15| To obtain eggs, breed the fish in groups (option A) or in pairs (option B). Group spawning is a long-term approach that is normally used for general maintenance of *N. furzeri* cultures. It requires only periodic egg collection from multiple spawning pairs but lacks direct feedback regarding clutch size. By contrast, pair spawning requires the immediate collection of eggs from a single pair and is used mainly for experimental purposes; this approach offers instant feedback and high control over clutches.

(A) Group spawning method ● TIMING 14 d

(i) Prepare spawning place for fish by filling a glass jar (15-cm height, minimum volume 0.6 l) with a 6-cm layer of wet boiled peat; peat protects the eggs better than glass beads during long-term exposure to adult fish. Use bottle-shaped jars with an opening of at least 8 cm in diameter (**Fig. 6**). The bottle shape prevents excessive spilling of the peat.

(ii) Add aquarium water to completely fill the jar and wait for the peat to settle to the bottom (5–10 min). Place the spawning jar inside the aquarium very slowly so that the water disturbance does not wash the peat out. Maintain at least 10 cm of water above the jar to enable free movement of the fish. Introduce a group of parental fish while maintaining maximum adult fish density (4 l per fish). Keep the sex ratio at 1:3 (male:female—e.g., 5 male and 15 female fish for an 80-liter tank). Fish will begin to enter and spawn in the jar within 1–2 d. Normally, fish eject at least some peat from the jar, which is a reliable sign that spawning has commenced. Siphon out any spilled peat to prevent fish spawning outside the jar.

(iii) *N. furzeri* tend to cannibalize their eggs. To minimize cannibalization, maintain an ~6-cm-thick peat layer to better conceal the eggs; keep the fish constantly satiated (continual presence of live food in the tank is especially beneficial during group spawning); as particular fish tend to specialize in cannibalizing eggs, remove these individuals from the tank; avoid dropping food into the spawning jar during feeding.

(iv) Swap individual male fish in the aquarium every 24 h or add an additional spawning jar per each additional three male fish to enable breeding of subdominant male fish. Change and dry the peat as described in Step 16A of the PROCEDURE every 2 weeks.

(B) Pair spawning method ● TIMING 3.5 d

(i) Separate female fish from male fish for 48 h to maximize egg yield.

(ii) Meanwhile, prepare a small (2 l, 15 × 10 × 15 cm) aquarium or tub (clear plastic food boxes work well; **Fig. 6**). Prepare a lid to loosely cover the container. Use a 0.5- to 1-cm layer of laboratory glass beads (see Reagents section) as a spawning substrate. Alternatively, use an aquarium setup without the substrate (**Box 2**).

(iii) Fill the spawning container with water. Use warm water (maximum 34 °C) for the start of spawning but let it naturally cool down to room temperature.

(iv) Introduce a single male and female into the tub. Maintain dim lighting and minimize activity in the vicinity. Cover the spawning container. Keep the pair in the spawning container for 2 h (no aeration needed)—this is normally sufficient for laying all the eggs that the female fish has ovulated. Return the fish to their home aquaria after spawning.
▲ **CRITICAL STEP** Pair *N. furzeri* regularly (every 2–4 d) to habituate them to handling and to start spawning immediately after being paired.

(v) After removing the fish, briefly check whether the pair actually spawned: hold the tank against a light source, place an index finger into the spawning substrate and move it slowly along the full length of the shorter side of the spawning container. A few (1–4) eggs should become dislodged from the substrate.

(vi) Wait for at least 5 h (preferably 12 h) before recovering the spawned eggs (the egg envelope hardens). Prepare a vessel filled with water for sieving the eggs from the spawning substrate, an empty Petri dish to hold the eggs and methylene blue solution (0.002 g/l).

Box 2 | Pair spawning of *N. furzeri* without substrate ● TIMING 2 h

1. Prepare the spawning tank using a 4–5-mm mesh to separate the bottom of the container from the spawning fish. Cut off the entire bottom of a plastic food box and replace it with mesh.
2. Insert this box into another box of the same size, and allow a gap of 15–20 mm between the two bottoms (**Fig. 4c**). This holds the spawned eggs out of the reach of the spawning pair.
3. When the mesh method is used, the initial 1–3 spawnings may not yield the usual number of eggs, but the fish soon become accustomed to the setup and spawn readily. To recover the eggs from the mesh-equipped aquarium, simply siphon them out using a Pasteur pipette (**Fig. 4g**) (cut the tip off).

(vii) Use a sieve with a mesh size of ~1 mm (e.g., a tea strainer, as the mean size of *N. furzeri* egg is 1.35 mm) (**Fig. 6**) to recover eggs from the spawning substrate by sieving the substrate through the mesh. Use the water current from a wash bottle to transfer the substrate to the seive (this minimizes substrate particle rubbing against the egg surface). Submerge the sieve in the water vessel so that the substrate does not spill over the edges of the sieve. Use slow, circular movements to wash the substrate away from the sieve. Check whether there are any eggs caught in the sieve (eggs are clearly visible to the naked eye).

(viii) Use a laboratory wash bottle (**Fig. 6**) to rinse out remaining eggs from the sieve into a large Petri dish. Expect ~20–120 eggs per female fish depending on female size and condition. Remove the washing water from the Petri dish by siphoning it out with a Pasteur pipette (**Fig. 6**), and replace it with the methylene blue solution.

? TROUBLESHOOTING

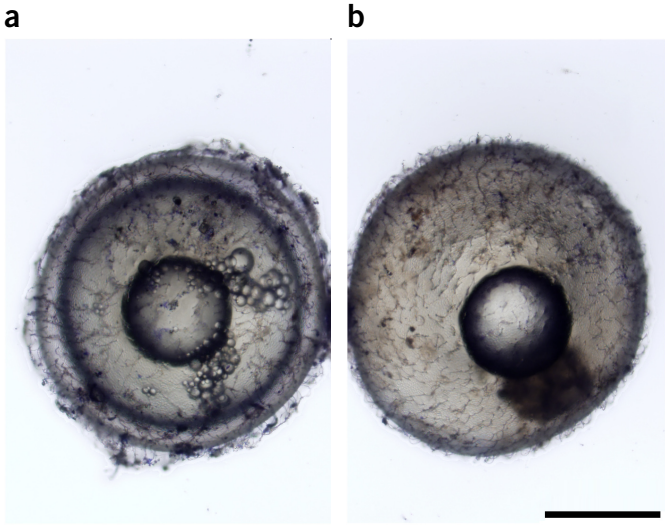

Figure 9 | Differences between a fertilized and an unfertilized *N. furzeri* egg. (**a**) A fertilized *N. furzeri* egg with a typical double-layered structure. (**b**) An unfertilized *N. furzeri* egg lacking perivitelline space. Scale bar, 0.5 mm.

(ix) Keep the eggs in the methylene blue solution for ~12 h (or 3 d when fertilized eggs will be incubated in water; see Step 16D of the PROCEDURE). Check the fertilization rate under a stereomicroscope with bottom illumination. Fertilized eggs have a double-layered structure with a perivitelline space (a layer between the chorion and the vitelline membrane) and remain clear (**Fig. 9a**). By contrast, after 12 h, the single-layered, unfertilized eggs start decaying and acquire a bluish, rather opaque coloration (**Fig. 9b**). Remove unfertilized eggs using a Pasteur pipette. The fertilization rate is variable, but the typical range is 70–90%.

(x) Incubate the fertilized eggs as described in Step 16B–D of the PROCEDURE.

Incubation of eggs ● TIMING 17 d to 16 months

16| To incubate group-spawned eggs in peat, follow option A. Incubate pair-spawned eggs in peat (option B), on top of peat (option C) or in water (option D). Incubation in peat is used for general maintenance of *N. furzeri* culture and in experiments not requiring continuous feedback on actual embryonic development. This method is most closely related to natural conditions. Incubation on top of peat incorporates dry (natural) incubation while simultaneously allowing for gross observation of development during an experiment. Incubation in water (or a modified aquatic medium) allows for both a high degree of control and observation of developmental processes in detail, and offers a tool for potential chemical manipulation of the embryos.

(A) Incubation of group-spawned eggs

(i) Prepare double-layered sheets of filter paper (or newspaper). Prepare a net with fine mesh size (e.g., a 0.15-mm mesh used to sieve BBS). Remove the spawning jar (or previously wetted peat potentially containing undeveloped eggs from Step 8 of the PROCEDURE) from the tank and pour all the peat with eggs into the net. Squeeze the net firmly by hand until the water from the peat stops dripping. The eggs will not be damaged as long as the peat is fine structured and free of hard particles (see Reagent Setup).

(ii) Empty the contents of the hand net onto the filter paper and wrap it up to form a solid package. Label the package immediately with the strain ID and date of collection using a permanent marker. Leave it at room temperature until the surface of the filter paper is dry; this usually takes 2–4 d.

(iii) Unwrap the package to check the dampness of the peat. If the peat has the correct moisture content, it will show no signs of water reflectance inside the peat grain structure (otherwise it is too wet) and will have a very dark brown hue (otherwise it is too dry); the peat surface directly adjacent to the filter paper should be just starting to turn pale (i.e., drier than rest of the peat ball). If the peat is still too damp, rewrap it and wait for an additional day or place the package in front of a fan (shortens waiting to ~1/5 of time).

(iv) Check for the presence of eggs in the peat. Separate the clump (the usual volume is 150–250 ml) into halves. If the spawning is successful, meaning that ~5 eggs are visible (eggs are plentiful), proceed to the next step. If no eggs are visible after 30 s of searching by stirring the peat repeatedly, spawning was unsuccessful. Repeat spawning (Step 15A), taking appropriate corrective measures.

? TROUBLESHOOTING

(v) Using a spoon, move the peat with eggs into a plastic zipper bag and seal the bag. Using a permanent marker, mark the bag with important details (the date of batch collection, strain ID). It is practical to additionally label the bag with descriptive information on egg density (e.g., high, intermediate and low) to enable feedback on approximate embryo survival later.

(vi) Place the bag in an incubator. Set the incubation temperature according to the desired speed of embryo development (17–28 °C). Keep in mind that the effect of incubation temperature is not fully deterministic (see INTRODUCTION). Set the incubation temperature to 27–28 °C to speed up development and to increase the proportion of escape (nondiapausing) embryos; eggs that skip all diapauses are ready to hatch within 17–21 d. Set the temperature to 22–25 °C to achieve erratic development; eggs that enter a diapause will develop unevenly for 2–16 months. To induce dormancy in most eggs, incubate the eggs at 17–20 °C for at least 4 weeks after collection; switch the temperature to 25–26 °C to induce most embryos to exit diapause simultaneously and achieve synchronized development. Expect the eggs to be ready to hatch within an additional 4–6 weeks (i.e., 8–10 weeks in total). (For an overview of husbandry steps and incubation approaches, see **Fig. 5**.)
▲ **CRITICAL STEP** Note that temperatures higher than 28 °C lead to successful embryo development[17], but problems ensue: the time window for successful hatching shortens considerably; the embryos exhibit hatching problems; and newborn fish often suffer from belly sliding (inability to fill the swim bladder and swim normally).

(vii) Check the moisture level in the bags periodically every 1–2 months. If the peat is losing its dark brown hue, it is starting to dry out and a few drops of water should be added; mix the peat thoroughly by flipping and turning the bag upside down after adding the water, and wait for ~1 h to allow even soaking of the peat. If too much water is added, mix in some new dry peat to decrease the overall moisture level.

(viii) Before hatching, check the developmental status of the eggs inside the bag, as described in Step 2 of the PROCEDURE.

(B) Incubation of pair-spawned eggs in peat

(i) Prepare 200–300 ml of peat (see Reagents section). Allow it to dry until a pale brown hue is reached (in an open container or by wrapping it in filter paper); it should have a slightly lower degree of moisture than required for incubation, because some fluid will be transferred with the eggs. Transfer the peat to a plastic zipper bag.

(ii) Use a Pasteur pipette to transfer the eggs from the methylene blue solution (from Step 15B(ix) of the PROCEDURE) to the bag. Transfer each egg with as little fluid as possible (e.g., transfer the eggs in groups) to avoid overwetting the peat. Ensure that the eggs are evenly distributed within the peat to limit the potential spread of any fungal infection. Flip the bag up and down and stir the peat to avoid clumping.

(iii) Wait for 1 h after transferring the eggs before checking the dampness of the peat. Add a few drops of water and wait for another hour if it is too dry, repeating as required. If it is too wet, add some dry peat and mix the contents of the bag thoroughly until the correct moisture level is achieved. Leave the bag open and place it in the incubator if the moisture level is only slightly higher than is appropriate. Check the dampness of the peat repeatedly over the next 1–3 d. When the correct moisture level is achieved, seal the bag and continue incubation as described in Step 16A(vi—viii) of the PROCEDURE.

(C) Incubation of pair-spawned eggs on top of peat

(i) Prepare a compartmented Petri dish (**Fig. 6**) and fill each cell halfway with peat of the correct dampness (Step 16A(iii) of the PROCEDURE). Press the peat with fingers so that its surface is firm; create a concave shape to keep the egg in the middle of the cell.

(ii) Use a Pasteur pipette to transfer the eggs from the methylene blue solution (from Step 15B(ix)). Place the eggs on top of the peat, a single egg per cell.

(iii) Cover the dish with a lid. Mark the lid with date and strain ID. Seal the lid with Parafilm or place the dish into a large plastic zipper bag (several Petri dishes can be placed in the same bag) to prevent rapid evaporation.

(iv) Start incubating the eggs as described in Step 16A(vi–viii) of the PROCEDURE. Note that incubation on top of the peat increases developmental speed compared with incubation inside the peat (especially at incubation temperatures >25 °C). However, eggs incubated on the peat surface often hatch poorly, and this approach results in a larger proportion of belly sliders, at least in some laboratories.

(D) Incubation of pair-spawned eggs in water

(i) Incubate the fertilized eggs in methylene blue solution (from Step 15B(ix) of the PROCEDURE) for 3 d, changing the entire volume of the solution daily by siphoning and replacing it.

(ii) On the fourth day after fertilization, prepare 5% sodium hypochlorite (household bleach), sterilized water and a vial with a minimum volume of 200 ml. Transfer the eggs together with the methylene blue solution to the vial (clutches can be pooled if allowed by the experimental design). Pour off the methylene blue solution. Add 200 ml of sterilized water. While gently stirring the water, add 1 ml of the bleach; the bleach not only sterilizes the egg surface but also partly removes fibrils covering the egg surface and makes the embryo more easily observable. Keep the eggs in the bleach solution for 5 min (use a timer).

(iii) Pour off the bleach solution. Add 200 ml of sterilized water and stir gently. Wait for 1 min.
(iv) While gently stirring the water, add 1 ml of the bleach. Wait for 5 min (use a timer).
(v) Pour off the bleach solution, taking care to remove it completely using a Pasteur pipette. Add 200 ml of sterilized water. Add 1 ml of sodium thiosulfate solution (5 g/l) to neutralize any remaining bleach. Wait for 5 min (use the timer).
(vi) Pour off the water containing the thiosulfate solution, using a Pasteur pipette to remove it completely. Add 200 ml of sterilized water. Gently stir and wait for ~1 min.
(vii) Pour off the water and add 200 ml of 10 mg/l gentamicin sulfate solution[63].
▲ **CRITICAL STEP** During incubation in water, *N. furzeri* eggs are more prone to infection with bacteria and/or fungus than during incubation in the peat. They must be treated with antimicrobials at the beginning of the incubation period and handled antiseptically thereafter[63].
(viii) Distribute the eggs with the solution into compartmented plastic Petri dishes, one egg per cell, using a sterile Pasteur pipette. The medium level should be ~1 cm. Seal the dish with Parafilm to prevent rapid evaporation of the medium. Place the dish into the incubator at 25 °C.
(ix) Using a stereomicroscope, check the developmental status of the eggs on a regular basis (e.g., 2 times per week). Wait for the embryos to reach diapause II (ref. 16) (at the age of 9–11 d post fertilization, optic lobes and optic cups are prominent and 32 somites are visible) or a more advanced stage (some dark or golden pigmentation in the eyes).
(x) Trigger the exit from diapause II by increasing the temperature to 28–30 °C for 48 h (the embryos develop to the prehatching stage within the next 8–10 d if the exit was triggered successfully).
(xi) Transfer the diapause II or postdiapause II embryos to damp peat (this transfer is important for *N. furzeri* eggs to hatch normally). Incubate the embryos as described for pair-spawned eggs in peat (Step 16A(vi–viii) of the PROCEDURE).

Shipping eggs ● TIMING 1–2 h

17| Select suitable eggs from the egg stock; use only eggs that are at least 3 weeks post fertilization (presumably in diapause II or ready to hatch). Do not use prediapause II (<9 d post fertilization) eggs for shipping because of higher sensitivity to handling stress and adverse shipping conditions than the resistant diapause II eggs.

18| Prepare a small (10 × 6 cm) plastic zipper bag with 20 ml of peat. Use the peat in which the eggs were originally incubated, if possible.

19| Use entomological tweezers to move dry incubated eggs into the bag. Use a Pasteur pipette to move water-incubated eggs into the bag with peat. Avoid making the shipping peat too wet by limiting the amount of water transferred with the eggs. Adjust the final dampness of the peat by adding some dry peat if necessary (Step 16A(iii) of the PROCEDURE).

20| Seal the bag. Prevent clumping of the eggs and achieve even distribution of the moisture by flipping and shaking the sealed bag. Label the bag with strain ID, date of the spawning and number of eggs.

21| Wrap the bag in bubble wrap and place it into an envelope. Ship the eggs.
! CAUTION For international shipping, follow applicable laws and regulations.
▲ **CRITICAL STEP** Do not send the eggs if a temperature above 4 °C cannot be guaranteed during the shipment. Low temperatures may kill the embryos.

Quarantine procedure for new fish and eggs ● TIMING 30 d

22| Keep any new fish coming to the husbandry facility quarantined for 30 d. Place the fish into a disinfected, completely isolated tank. Use only dedicated equipment for any manipulation. Do not allow any direct contact of the new fish with the established stocks. Avoid indirect contact through, e.g., water hoses, water buckets, splashing water or wet hands. Assign a different person to take care of the quarantined fish than for established stocks, if possible.

23| Observe the behavior and appearance of the fish for at least 10 min each day (assign an experienced person). Prepare a protocol with date, time and results of the observation; describe the appearance of the fish and their behavior to obtain a time line of the development of any potential problem. Consult with a specialist if any problems are suspected; wild-caught fish are often heavily parasitized, but many metazoan parasites pose no threat of a direct infection to other fish in the facility because of the complex life cycle of both the fish and their parasites.
▲ **CRITICAL STEP** Do not apply any unspecific chemical treatment (e.g., acriflavine, FMC (formaldehyde–malachite green solution) methylene blue, malachite green or broad spectrum antibiotics) without observing symptoms of a real disease, as this would pose an additional stress load for fish in a new environment.

Box 3 | Everyday preventive quarantine ● TIMING 5–120 min

1. Keep tanks with the fish isolated from each other, as the use of live and frozen foods presents a risk of infection with a pathogen. Disinfect each tank with sodium hypochlorite solution before introducing any different group of fish. Add 10 ml of sodium hypochlorite per 100 l of water directly to the tank. Keep the filter in the tank and running. Wait for at least 30 min. Remove the filter and wash it thoroughly under running water by squeezing it repeatedly for at least 5 min. Empty the tank completely and refill it with water. Return the filter and turn it on. Add 10 ml of thiosulfate solution per 100 ls of water. Wait for at least 1 h. Empty and refill the tank. Ensure that the water is free from chlorine using a chlorine test strip.
2. Use only disinfected equipment for any manipulations of the fish. Prepare saturated NaCl (kitchen salt) solution by adding 360 g of salt per 1 l of water. Use the solution for disinfecting the manipulation equipment such as hand nets. Equip the fish room with a vessel and fill it with the salt solution. Keep all hand nets constantly wholly submerged in the solution, and return each of them after use. Ensure that the solution remains saturated (some amount of salt is lost with each use of the hand net) by adding salt to maintain some undissolved salt on the bottom of the vessel. The use of salt poses no threat to the fish, and the solution is more stable than bleach.
3. Avoid contact of tank water with the water filling hose (keep it outside the tank water during filling), feeding scoops, tweezers and pipettes to minimize the risk of pathogen transfer between tanks. Use a dedicated siphoning/water change hose for each tank, if possible. Minimize the extent of wetting hands during manipulation in tanks whenever possible.
4. Boil (or autoclave) spawning and incubation substrates after each use to remove potential pathogens and to prevent strain contamination.

24| Keep collecting eggs using the group-spawning method (Step 15A of the PROCEDURE), which results in the least handling stress. Incubate the collected eggs (Step 16A of the PROCEDURE), but hatch them to found a new strain only if the parental fish are proven to be healthy. Maintain everyday preventive quarantine (**Box 3**).

Oodinosis treatment

25| If oodinosis is suspected, examine a live individual fish under a strong external lamp. Place the fish in a small aquarium and inspect it visually. Morphological symptoms: search for very small (0.1 mm) but distinct yellowish spots on the fish's surface (**Fig. 3b**), in contrast to the surface of a healthy fish (**Fig. 3a**). These are found throughout the whole body surface but are more concentrated above the eyes (eyebrows) and on the very proximal part of the dorsum in front of the dorsal fin. Transparent fins of the female fish appear to be dotted when looking at them with the light source behind the fish. Behavioral symptoms: in the early stages, fish keep their fins spread, but later the fins are folded. Oodinosis outbreaks are more common in soft water (conductivity <300 μS/cm^2) as compared with hard water (>500 μS/cm^2). Juvenile fish appear to be more resistant to the disease than are adults. Note that fish continue eating until the very final stage of infection. When symptoms of the disease are recognized, all fish in the tank are already infected. To treat oodinosis, use option A, short-term bath, or option B, long-term bath. When performing the long-term bath (option B), fish can be treated directly in the infected aquarium, so this option should be used if space is limited. However, the long-term treatment increases the risk of spreading the disease to other aquaria and stops the reproduction of the fish during the treatment (fish cease spawning, eggs suffer high mortality).

▲ CRITICAL STEP Note that treatments for disease may have longer-term effects that interfere with functional assessments of experimental fish.

▲ CRITICAL STEP The use of remedies other than salt treatment to cure oodinosis is not recommended. Other remedies present a high toxicity risk. Never use copper sulfate, as its toxicity is especially high for *Nothobranchius* spp.

(A) Treating oodinosis with a short-term bath ● TIMING 30 min

(i) Prepare a 10% solution of NaCl and aquarium water (100 g salt per 1 l water). Prepare a new, well-aerated, infection-free tank; two disinfected hand nets (submerged in saturated salt solution for at least 1 h); and two small (2-l) disposable plastic tubs.

(ii) Pour the 10% salt solution into one of the plastic tubs to a water depth of 2 cm. Transfer all fish from the infected aquarium to the second plastic tub. Using one of the hand nets, begin placing the infected fish into the 10% salt solution in groups of 3–5 individuals. The fish will become very agitated at first. The salt solution is too concentrated for the fish to swim in normally, so they will lie on their sides after 5–10 s.

(iii) Observe the fish. Wait until their opercular opening (breathing) stops and they remain motionless (~10–30 s after transfer). Wait for another 5 s and then quickly transfer all motionless fish to the new aquarium, using a second hand net.

▲ CRITICAL STEP Use two separate hand nets: one net for transferring fish from the salt solution to the clean aquarium, and another for removing fish from the infected aquarium.

(iv) Check the fish treated with the salt solution under a strong light (e.g., 60-W light bulb above the tank). Treated fish should be visibly clear of spots immediately after treatment, as the concentrated solution causes an osmotic shock that kills the parasite. Although the fish may initially remain lying on the bottom and breathing heavily after the transfer, they will start swimming soon and will fully recover. Maintain usual care after the treatment.
! CAUTION Apply for ethical approval to perform the treatment. Although the procedure minimizes the overall amount of stress to the fish, fish are temporarily distressed during treatment. Apply for ethical approval in advance, as treatment of infected fish needs to be performed within days.

(B) Treating oodinosis with a long-term bath ● TIMING 3 d

(i) Perform a 50% water change and then add 40 g of loose NaCl per 10 l water (water conductivity up to 7,000 µS/cm^2 is well tolerated) to the aquarium. Maintain this concentration for 3 d by omitting all water changes while adhering to the normal feeding regime. Keep the filtration on.

(ii) Revert back to the normal water change schedule after 3 d. Disinfect all equipment that came into contact with the infection using saturated salt solution (e.g., hand nets; see **Box 3**).

Dropsy treatment ● TIMING 5 min

26| Observe the appearance and behavior of fish to diagnose dropsy. The body is generally swollen, with scales protruding upward (**Fig. 3d**). A sick fish has considerably decreased activity and stops eating. Typically, only a single or small number of individuals from the stock show the symptoms.

27| The condition is untreatable. Discard the fish with the disease and follow recommendations for fish care for the remaining fish.

Treatment of unspecified infections ● TIMING 3 d

28| Infected fish have decreased activity and appetite, clamped, whitish fins and protruding eyes (exophthalmos) (**Fig. 3c**). Use NaCl in the same manner as for long-term oodinosis treatment (Step 25B) to treat an unspecified infection. Add one large sea almond (*T. cattapa*) leaf per 20 l of water. After 1–2 d, the leaves will leach humic and tannic acids (dyeing the water brown) with antibacterial and antifungal inhibitory activity. Feed the fish normally. Keep the filter running.

29| Return to the usual water change schedule after 3 d of salt treatment. Keep the leaves in the aquarium until they naturally disintegrate (~4 weeks). Our experience shows that this treatment results in ~80% success in treating infections.

***Glugea* spp. treatment ● TIMING 3 d**

30| Look for parasitic xenomas to diagnose *Glugea* spp. These are manifested as white cysts up to 5 mm in diameter found inside the abdominal cavity on various organs (**Fig. 4**).

31| Discard all infected fish, their eggs and any peat used to incubate them. Thoroughly sterilize all equipment using 10 ml/100 l sodium hypochlorite solution (**Box 3**).

32| Quarantine any new fish or eggs coming to the laboratory.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Eggs do not hatch	Lack of stimuli (oxygen concentration, temperature and absence of humic substances)	Forced hatching. Using a Pasteur pipette, transfer the unhatched eggs to a vial that is 5–8 cm wide. Add hatching water to a depth of 2–3 cm. Cover the eggs with a 0.5-cm layer of the glass beads used for pair spawning (see Reagents section). Wait for 0.5–1 h. Most eggs (80–90%) should hatch (provided they are still viable, i.e., not overdue or damaged)

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Juvenile belly sliding	Unknown/low ambient oxygen concentration	Increase the oxygen concentration in the water. Prepare one oxygen tablet per 1 l of hatching water. Crush it into a fine powder. Add the powder to the water. Crushing the tablet further increases the surface area for the chemical reaction and maximizes oxygen concentration at a single time, which may further aid the fish in filling in their swim bladders[62]. *Note:* Do not increase the dosage. An oxygen concentration that is too high can be lethal to the fish
11	Juvenile cannibalism	Malnutrition	Increase the food ration. Cannibalism is extremely rare if fish are fed properly
15B(viii) and 16A(iv)	Low egg production	Insufficient feeding	Increase the food ration for parental fish. Feed fish twice a day *ad libitum* and provide higher-quality food (ranked from best to worst in terms of egg production: live *Tubifex* worms > live bloodworms > live mosquito larvae > live glass worms > frozen bloodworms > live zooplankton > frozen glass worms > frozen fairy shrimp > frozen zooplankton > dry food)

● TIMING

Steps 1–5, hatching (shipped) eggs: 6–12 h
Steps 6–12, raising juvenile fish: 3–4 weeks
Steps 13 and 14, care of the adult fish: 3–9 months
Step 15A, group spawning method: 14 d
Step 15B, pair spawning method: 3.5 d
Step 16, incubation of eggs: 17 d to 16 months
Steps 17–21, shipping eggs: 1–2 h
Steps 22–24, quarantine procedure for new fish and eggs: 30 d
Step 25A, treating oodinosis with a short-term bath: 30 min
Step 25B, treating oodinosis with a long-term bath: 3 d
Steps 26 and 27, dropsy treatment: 5 min
Steps 28 and 29, treatment of unspecified infections: 3 d
Steps 30–32, *Glugea* spp. treatment: 3 d
Box 1, individual housing of *N. furzeri* in a recirculating system: 3–9 months
Box 2, pair spawning of *N. furzeri* without substrate: 2 h
Box 3, everyday preventive quarantine: 5–120 min

ANTICIPATED RESULTS

By using this protocol, we achieve hatching and proper juvenile development in 70–90% of embryos. Sexual maturity is reached in 3–4 weeks; full egg production (40–80 eggs per 2 h spawning sequence, repeated every second day) is achieved in female fish at the age of 8 weeks (after rapid growth ceases). Fish longevity depends on the laboratory population; in our facility, wild-derived populations live 4–8 months and fish die from senescent deterioration that is macroscopically and histologically apparent. Juvenile mortality depends on rearing density but is negligible (<10%) if all measures described in this protocol are followed. We reiterate that the successful husbandry of *N. furzeri* stems from appreciating their difference from other fish laboratory models—most notably the need for higher rations of high-quality food (live or frozen) and the stochastic nature of their embryonic development. *N. furzeri* are tolerant to a wide range of water chemistry.

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AUTHOR CONTRIBUTIONS M.P., R.B. and M.R. developed the protocol over the last 10 years. M.R. initiated the paper. M.P. drafted the protocol. R.B. prepared photographic documentation. All authors contributed to the final text.

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