LABORATORY MANUAL FOR CULTURING *N. furzeri*

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An updated protocol has been published which surpasses this one with respect to laboratory care: Polačik et al. Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*. Nature Protocols 11:1396–1413, 2016, http://dx.doi.org/10.1038/nprot.2016.080.

Contents

1 *N. furzeri*: natural history

Nothobranchius furzeri was discovered by Mr. R. E. Furzer and Dr. W. Warne in 1968 and 69[1]. The fish were collected from the Sazale Pan in the Gonarezhou Game Reserve in modern day Zimbabwe (formally Gona-re-Zhou in Southern Rhodesia). *N. furzeri* was found together in the same pan with *N. orthonotus* and the lungfish *Protopterus annectens*. Jubb reports that fish were also found in an adjacent pan.

Some eggs of *N. furzeri* were smuggled out of Zimbabwe to the USA in 1970 to Robert Parle (AKA)[2]. From these eggs viable fish were hatched and distributed in the hobby. Some eggs or fish eventually made their way to Rosario La Corté in New Jersey who sent 24 eggs to Walter Foersch in Germany[3]. From the eggs sent to Foersch, only two fit pairs were obtained.

While La Corté maintained his line for many years (pers comm), while many other hobbyists lost theirs, it does not seem that his line has continued in any other form other than the pair that was raised by Foersch. From the various hobbyist resources[4] all fish currently in the hobby are most probably descended of the fish raised by Foersch.

From the various hobbyist informations, it seems that the *furzeri* in the hobby manifested two clear phenotypes: a short-lived strain living about three to four months; and a longer-lived strain that reached about six months[5]. Only the short-lived strain appears to have persisted in the hobby from Foersch's line. But fish in the possession of Alexander Dorn (presumably from a DKG import from Dr. Brian Watters, AKA) are capable of attaining six months, although rarely. This is a recent manifestation, suggesting contamination of the hobby strain by wild long-lived stock from Mozambique or a mutation in the allele pertaining to the manifestation of the short-lived phenotype.

Foersch[3] and Luehring[6] observed that the fish lived a short period of time (only four and a half months), grew rapidly and had an erratic egg incubation period. These observations are key to the successful breeding and maintaining of *N. furzeri*.

1.1 The natural habitat

Gonarezhou Game Reserve lies on the steep escarpment separating the lowveld and the highveld plains of southern Africa[7]. Its approximate 5 180 m^2 is between 300–600 m above sea level. Its climate between October and February (the wet season) is described as uncomfortably hot by Jubb.

It receives between 250–400 mm of rain per year; but may not receive any rain some years. It is described as being semi-arid.

The park is bordered by the Nuanetsi River in the South (that drains into the Limpopo) and the Lundo River in the North (that drains into the Sabi and then Save

[1] JUBB, R. A. (1971) A new *Nothobranchius* (Pices, Cyprinodontidae) from Southern Rhodesia. *J. Amer. Killifish Assoc & Killi Notes*, **8**(1):12–19

[2] PARLE, R. (1970) Two new *Nothos* from Rhodesia. *AKA Killi Notes*, **3**(6):15–21

[3] FOERSCH, W. (1975) *Nothobranchius furzeri* (Jubb 1971). *J. Amer. Killifish Assoc & Killi Notes*, **8**(11):315–321. Translated by Alex Thiermann from the original article published in the April 1975 DATZ German aquarist magazine.

[4] Most notably Lee Harper and Robert Ellerman (both AKA) who maintain a vast store of old killifish related publications.

[5] BELLEMANS, M. (2005) Nothobranchius *information center*. http://users.pandora.be/\marc.bellemans.

[6] LUEHRING, D. (1975) My experiences with *Nothobranchius furzeri*. *J. Amer. Killifish Assoc & Killi Notes*, **8**(11):322–324.

[7] JUBB, R. A. (1981) *Nothobranchius*. TFH Publ., NJ USA.

Rivers). The central watershed drains into the Guluene and Chefu Rivers that are tributaries of the Chingovo River of Mozambique. The Chingovo River degenerates into a series of swamps, pans and lakes that lead into the Limpopo valley.

The area is infested with tsetse flies. Jubb notes that the pan from which the fish come from is used as a watering hole by elephants and other game, and is rich in organic material due to fecal matter deposited by the game.

1.2 Distribution of *N. furzeri*

In his 1981 publication[7], Jubb indicated that *N. furzeri* was only known from pans at the headwaters of the Guluene River in Gonarezhou. He further speculated that the fish may be found in the swamps and pans associated with the Chingovo River in Mozambquie; and possibly the Save River system.

In 1999 Trevor Wood (BKA), Peter Riley (BKA), Johan Ippel (S. African), Pieter Kearney (S. African) and Jan du Plooy (S. African) went on a killifish collecting expedition into Mozambique[8]. As well as finding several new locations of black *N. rachovii* and red and green *N. orthonotus* they discovered populations of a fish reminiscent of *N. furzeri* along the Limpopo River just south of Mabalane. The MOZ 99-4 population, instead of having a lemon yellow band in the caudal fin, had a solid red tail along with more intense red in the anal fin. The MOZ 99-5 and 99-6 populations were similar to the MOZ 99-4. Both strains proved difficult to spawn successfully in captivity and the MOZ 99-5 and MOZ 99-6 strains appear to have been lost. The MOZ 99-4 persevered for several generations wherein, it would produce fish of the Gonarezhou phenotype occasionally[9]. This strain is very rare in the hobby. In 2000 there was severe flooding in the Limpopo system, so it is likely that the genomes of the MOZ 99 *furzeri* populations are no longer extant in the form they were in 1999 due to population mixing.

In 2004 three expeditions were made to Mozambique: Cellerino & Valdesalici (MZM 04) of Italy; Brian Watters (MZ 04); and again Brian Watters (AKA), Rudolf Wildekamp (Netherlands), Barry Cooper (AKA), Johan Bornman (S. African), Johan Ippel, Riaan Ippel and Johan Jordaan (S. African) (MOZ 04).

Watters et al discovered *N. furzeri* west of the Limpopo along the Mazimechopes River (MZ 04-2, MZ 04-3 and MOZ 04-13)[10]. The MZ 04-2 population contained both red and yellow individuals. The MOZ 04-13, to the author's knowledge, only manifests the yellow tailed phenotype.

Cellerino & Valdesalici traveled further inland and found *N. furzeri* all along the Limpopo River on the eastern bank, as well as inland where the Chefu River drains into the Chingovo River in Zanave National Park. Collections along the Save River also revealed *furzeri*. The red and yellow morphs were widespread across all drainages, and most pans held both phenotypes.

The full natural range of *N. furzeri* is not yet established, nor are matters of its wild ecology and physiology.

[8] WOOD, T. (August 2000) Trip to Mozambique, 1999. *BKA Killi-News*, 105–119

[9] Richard Cox (BKA), pers comm

[10] WATTERS, B.R. (2004) *Distribution of* Nothobranchius *Species/populations by Country.* AKA website: www.aka.org/pages/libary/Notholoc.html.

2 Caring for adult fish: tank setup and maintenance

2.1 Tank setup

A 45 $\times$ 25 $\times$ 30 cm tank (≈ 30L) is adequate for 10 to 15 adult fish between 4 and 8 cm in size. The larger the tank, the more stable the water quality and the larger the fish will grow.

With daily 10–20% water changes filtration is not needed. As it may not be possible to do such water changes every day, the presence of a filter is beneficial. A small sponge or box filter with a mild trickle of bubbles is adequate. The free standing Hydro-sponge filters[11] make maintenance and fish counting easier. A temperature of ≈ 25.0°C (within the limitations of the thermometer and thermostat) is maintained for all experiments and controls unless otherwise stated.

The tank need not have any decoration. Some Java moss (*Taxiphyllium* or *Vesicularia* species) can be added to offer some hiding space for harassed fish, and aid in water purification by assimilating ammonia/ammonium produced by the fish.

The fish are unparticular about pH, but large rapid pH swings are best avoided. A pH between 6 and 8 would appear adequate based on available data. A rapid swing from 7 to 6 can impede the biofilter efficiency by inhibiting the bacteria mediated conversion of ammonia to nitrite and nitrate, which can cause an accumulation in ammonium concentrations. A corrective water change to rescue the declining pH could then cause the ammonium to convert to ammonia, which may then be at lethal concentrations. The fish themselves may develop blood acidosis in response to the low pH.

In water devoid of calcium and magnesium the pH can be very unstable. This is easily remedied by the use of commercial water buffers, but these can have detrimental health effects by disturbing osmotic balance in the fish due to rapid chances in the conductivity of the water that is often associated with such buffers. Wright Huntley (AKA, pers comm) and Barry Cooper[12] suggest the use of Equilibrium™ (a product by Seachem) that supplements essential minerals (including potassium, calcium and magnesium) and buffering capacity.

For many years the author simply included crushed coral or shells in the box filter. This was able to maintain a more or less stable pH. Most pet stores will stock crushed coral or shell, or substances such as argonite that are essentially calcium and magnesium carbonate.

Velvet (*Oodinium* species) are parasitic pests often associated with soft water, low pH or generally poor husbandry conditions. Its treatment is discussed in Section 4.5, page 12. Table salt at a dose of 1 teaspoon per 3.8 L is normally suggested as a prophylactic against velvet infection, but an excess of sodium in water with a very low total dissolved solids (tds) reading can have detrimental effects on physiology and health by disturbing the electrolyte balance and osmotic processes of the fish. This worry is supported by veteran *Notho* breeder Ian Sainthouse (BKA, pers comm), who reports fish raised in the absence of table salt dosing live longer than those whose tank's were dosed. Velvet outbreaks are rare in water with a high calcium and/or magnesium content.

[11] Manufactured by Aquarium Technology, Inc, www.4fishstuff.com. A similar model by another brand would suffice presumably.

[12] COOPER, B. J. (2003) Managing water conditions with a recirculating system—a case study. *J. Amer. Killifish Assoc.*, **36**(1):13–20.

2.2 Feeding

The staple diet for adult *N. furzeri* in the laboratory is *Chironomus* larvae, either fresh or frozen. Only as much should be fed as what can be consumed within 10–15 minutes. With experience it becomes possible to estimate what quantity this is for a given number of fish of certain size.

The *Chironomus* should be as fresh as possible and of high quality. Blister packs (where the *Chironomus* are frozen into a little cube for easy feeding) is best avoided, as in the production process the *Chironomus* is frozen and then thawed and then frozen again, at the cost at some delicate nutrients and vitamins [13]. This freezing and thawing process can allow bacterial pathogens to proliferate in the food, posing a health risk to the fish. The fresher the *Chironomus* the better and safer. Any *Chironomus* that has been left unfrozen for a time should be discarded rather than risk bacterial infection and food poisoning.

A local source of live and washed *Chironomus* is best. This can be frozen in portions suitable for feeding.

If possible add the frozen *Chironomus* directly to the tank[13]. If this is not possible, thaw only as much as what will be used in one feeding session.

To thaw the *Chironomus* place a frozen portion in a filter or strainer through which the *Chironomus* cannot pass and run cool water over it.

At no time should there be uneaten food left rotting at the bottom of the tank. Some snails may assist in scavenging uneaten food but generally cannot handle *Chironomus* larvae. It is best to siphon away any food leftover after feeding.

2.3 Intraspecific aggression

In general groups of *N. furzeri* coexist peacefully, but during the week or two during which they mature there may be some fighting as an invisible hierarchy is established. Male fish are best sorted into groups based on size. Females are not normally aggressive but exceptions can occur. Females of *N. melanospilus* can be more aggressive than males of the same species and care must be taken to protect males from the females and from each other.

In the case of a particularly aggressive male he should be removed or another intervention attempted. The normal intervention is to remove the male for a short period, rearrange the tank and reintroduce him to the tank. In this case he has lost the home-ground advantage. A new hierarchy will form which may be more peaceful than the previous one.

The use of large spawning vessels and an otherwise bare tank (i.e. no fixed territorial markers) can greatly reduce aggression between the individual fish. This is not the case with some strains of *N. orthonotus* which can only be described as malicious. This may be the influence of tank size more than an ingrained behavioral trait. 10 *N. orthonotus* Limpopo River MZM 04-3 were able to peacefully coexist the duration of their natural lifespan in an area of 45 $\times$ 45 cm whereas, a group of 26 Ceramica 2 MT 03-2 were not, instead murdering each other right down to the last fish. 20–30 *N. furzeri* can easily be maintained in this space although there may be a reduction in growth due to cramped quarters and competition for food.

[13] BELLSTEDT, D. U. (1997) Feed your discus a proper diet. *South African Fishkeeping* **2**:11–13. (This magazine is defunct and back issues are unavailable. The author can be contacted at dub@sun.ac.za.)

3 Spawning *N. furzeri* and other *Nothos*

Nothos are eager spawners. It is difficult to stop a male from wanting to spawn, and a receptive female from complying with him. A well conditioned and gravid female will actively seek out a male with whom to spawn with. All that is required on the part of the keeper is to provide a suitable substrate over which the fish can spawn so the eggs can be retrieved.

There are various materials that can be employed as a spawning substrate. The only two that will be discussed here are peat and fine silica sand.

3.1 Peat

3.1.1 Peat and its preparation

Peat can be broadly defined as anaerobicly decomposed organic material. This material can consist of peat moss leaves, heather leaves and twigs, grass leaves and stems, or even fern leaves and fronds depending from where the peat originates. The peat from Canada and Michigan USA is considered the best peat to use. It is composed of moss leaves and stems and is of a soft texture but, like other peats, is very acidic (able to drop the pH of soft water to 4 overnight) on account of the various organic weak acids contained therein.

Due to the acidic nature of peat it has to be suitably treated before use. This can be by boiling several times in the presence of an alkali such as sodium bicarbonate, calcium hydroxide or carbonate. The boiling also drives out the air in the peat enabling it to sink in an appropriate period of time.

If the goal is to collect the boiled peat extract for later use (see section 4.1, page 10), sodium bicarbonate should definitely not be used in the case of using water with a low level of tds. The increase in sodium concentration in very low tds water can be fatal for the same reasons explained in Section 2.1, page 3.

The quantity of peat to be prepared depends on how much can be used in a given period of time. Wet submerged peat removed from an anaerobic environment will begin to decompose and may rot, becoming unsuitable for use. Preparing as much as one can use in a week or two can be safely stored submersed. Alternatively the excess water can be squeezed from the peat and the peat stored in a plastic bag at 4°C until needed.

Jiffy peat pellets need not be boiled with alkali as it already contains some lime to neutralize acidity. The # 703 Jiffy peat pellet[14] is suggested. This is a soft, fine textured peat with a pH of 6.8. Jiffy is product of Grund, Norway.

Peat need not be boiled. Simply pouring very hot or boiling water over the peat may be sufficient. This process may need to be repeated several times. The alkali is best added directly to the dry peat.

3.1.2 Using peat

The soggy peat is best placed into a container with a snap-fast lid through which a hole has been cut, that is about 1.5× the maximum size of the fish to be spawned. In spawning, the fish will thrash about in the container and without the lid (with the

[14] WARNER, E. (1977) *Success with killifish.* The Palmetto Publishing Co. St. Petersburg, Florida.

relatively small hole cut through it) the peat would be strewn all over the tank. To the container, a stone is added to weight the container so it can be sunk into the tank and not easily overturned. There is some speculation that the stone or stones also give the fish something hard to spawn against which aids in the female expelling her eggs. The container can be sunk slowly into the tank after a waiting period for the peat to settle in the tub.

Some fish may not readily take to the peat container and may require a period of training to accept the peat container. This is facilitated by using a large shallow dish (initially without the lid). Once the fish have identified this as the spawning vessel the large dish can be switched for a smaller more peat efficient container.

Some fish are more stupid than others. Gonarezhou *N. furzeri* will rapidly take to a peat container, while *N. kilomberoensis* may struggle for a long time before finally accepting the peat container as a spawning substrate.

In general wild fish rapidly accept the artificial spawning substrate and wild males (which are more fiercely territorial than captive bred fish) will take up station above it, defending it from conspecifics.

There is some evidence suggesting that long-lived *Nothos* are stupider than short-lived *Nothos* in respect to identifying the spawning container. This hypothesis is based on the observation that long-lived *N. furzeri* that perform poorly in active avoidance and spontaneous locomotion assays also take longer to learn what the spawning vessel if for, based on the lower numbers of eggs collected from these vessels compared to short-lived fish that excel both in the assays and in egg production.

3.1.3 Collecting eggs spawned over peat

Harvest peat at least once every two weeks, else the peat may begin to rot and kill many eggs. It has been hypothesized[15] that the rotting peat depletes the oxygen levels within it forcing the eggs into a prolonged diapause I.

The peat and eggs are harvested by pouring the contents of a peat container into a fine mesh net. Some sources suggest giving the peat ball a firm squeeze but this may damage freshly laid eggs or even old eggs should there be hard or sharp bits in the peat. It is safer to force the peat into the bottom of the net and then flip the contents out onto newspaper or paper toweling in a soggy state.

The peat is wrapped up in the newspaper or paper toweling and the package clearly labeled. Peat wrapped up in two layers of newspaper will dry to the proper consistency within two to three hours. This consistency is often described as being moist like pipe tobacco. In the case of Canadian peat the peat should feel damp but have no obvious sogginess. When squeezed between the fingers a trace of water should emerge. This peat must be “fluffed” up to break up peat clumps and so ensure uniform aeration. The peat is then placed into a plastic bag, labeled with the species name, strain and collection date.

Some people advocate the use of ziplock freezer bags but these are made from very thick plastic that does not allow any air exchange which may lead to suffocation of the eggs therein. Thin sandwich bags loose too much moisture too quickly. Standard polyethylene fish bags are the best. Seal the peat inside with some air.

[15] WATTERS, B.R. (1998) *Killietalk, subject:* N. rachovii http://fins.actwin.com/killietalk/month.9807/msg00035.html

Peat placed on paper toweling may take 12 to 24 hours to dry to the proper consistency but can otherwise be handled the same.

Caring for incubating eggs will be discussed in Section 3.4.

3.2 Sand

3.2.1 Preparing sand

One of the virtues of sand is that it is easy to prepare. Simply pour the sand from the bag into the sieve you will use to retrieve the eggs and sift it to the appropriate particle size, keeping the sieved sand and discarding that which does not go through the sieve.

The sieving process may need to be repeated several times as some large particles are always forced through by the weight of the sand.

3.2.2 Using sand

Sieved sand can be placed into a dish or bowl without lid and submerged into the fish tank. The sand need only be 1 cm deep (any more and there may be complications in sifting the sand and collecting the eggs).

Nothos have no problem identifying the sand as a spawning substrate after a brief learning period.

The male attracts the female with flared fins and then the two descend onto the sand. Here the male will wrap his dorsal fin over the female and the spawning will take place. A second male may sneak in and try to fertilize the eggs simultaneously. When the pair leaves the substrate the female will flick her anal fin and bury the eggs.

The eggs are difficult to observe among the semi-transparent sand as compared to peat. The enables the eggs to escape the hungry eyes of the other fish.

After a few days of spawning the sand can be retrieved and sieved.

3.2.3 Handling eggs spawned over sand

In sieving sand, the sand is poured as a slurry into the sieve resting in a tub of water of the same temperature and tds as the spawning tank. With a gentle up-down motion, sieve the sand from the eggs.

To collect the eggs, invert the sieve over a second tub or sheet of tinfoil and firmly bang the sieve onto the tub or tinfoil sheet to free the eggs from the sieve. If over a tub, eggs still clinging to the sieve can be washed free using water of same temperature and tds as the spawning tank. If tinfoil is used eggs clinging to the sieve will need to be manually removed.

The eggs resting in water (of same temperature and tds as that of the spawning tank) in the tub are gently transferred to damp peat in petri dishes with blunt forceps. The eggs can be flicked off the forceps (rather than forced onto the peat) by striking the forceps on the side of the petri dish with the egg over the desired area of peat.

For tinfoil, the eggs are rinsed off (with water of the same temperature and tds of that in the spawning tank) into the bottom of a petri dish and the water carefully siphoned away. Damp peat is gently placed on top of the eggs. A second petri dish bottom is then placed upside down on top of the first dish, and with the quick rotation of the wrist, the dishes are inverted bringing the eggs to the surface on top of the peat.

The eggs are then manually sorted to ensure no eggs are in contact or clumped together, as if one of the eggs dies it can kill those around it. The eggs have to be inspected daily for the first few weeks, and dead fungusing eggs removed.

The petri dish is tightly sealed with parafilm to guard against dessication.

Instead of storing the eggs on peat they can be retained in water with some methylene blue (5 mg/L), peat[16] or sea almond *Terminalia catappa* leaf extract to deter infection and dye dead eggs so they can be easily identified and removed. (The peat and sea almond extract may need to be diluted for optimum use. The extract should be a pale brown or yellow respectively.)

Eggs stored in water will develop faster. At 27°C and above, they will develop in as little as three weeks. These eggs may spontaneously hatch in the water but can otherwise be forced to hatch as described in section 4.1, page 10. Eyed-up eggs can be moved to cooler temperatures and kept in the dark for a short time till one is ready to wet them.

3.3 The advantages and disadvantages of sand and peat

In spawning the fish over peat there is no need to sort the eggs as per sand spawning. However, in using sand and manually sorting the eggs onto peat a better estimate of the total number of eggs and fertility of fish can be attained.

Some pairs may be less productive than others. As sand spawning is more labour intensive it may be wise to employ peat and allow the fish to spawn for one or two weeks rather than sieve sand every other day. Alternatively, should the progeny of the fry be of high value spawning over sand offers more control. Both sexes can be separated from each other and conditioned prior to spawning to boost the number of eggs per spawning session. This is a reliable method to obtain viable eggs from old fish where male and female fertility is low.

In peat the development of the eggs is difficult to track and requires sifting through the peat to find eggs to observe. In spawning the fish over sand and placing the eggs on peat to develop makes observing egg development simple. Freshly laid eggs in peat are easier to find and these can be transferred from the peat to petri dishes, on top of peat for easy observation.

As the eggs often develop in a staggered fashion, placing the eggs on peat means that eggs that are ready to hatch can be easily identified and hatched, rather than allowed to languish in the peat where the resting fry may perish after exhausting its resources.

3.4 Caring for incubating eggs

Eggs can be stored between 20 and 24°C[17]. Lower temperatures slow development and warmer temperatures accelerate it. Also effecting development is oxygen supply and moisture of the peat. The moister and better aerated the peat the faster the development. Eggs in very moist peat which is clumped up will develop slower and erratically,

[16] KATZ, D. (2002) Water incubation of South American annual killifish eggs. *J. Amer. Killifish. Assoc.* **32**:137–145.

[17] WATTERS, B.R. (2005) *The Genus* Nothobranchius. AKA website www.aka.org/pages/libary/nothodoc.html

with the eggs lying along fissures in the peat developing much faster than those inside the peat ball.

The eggs of *N. furzeri* can stand temperatures down to at least 4°C. At and above 27°C the eggs develop rapidly being eyed-up in two to three weeks when incubated on top of peat in petri dishes. For *N. guentheri*[18] the eggs take in excess of 400 days to develop at 17°C 92 days at 19°C, 34 days at 22°C, and 14 days at 26°C when incubated in water. Factors affecting egg development are reviewed by Vitek & Kadlec[19].

Developed eggs can be moved to cooler conditions to await hatching or hatched then and there. At such high temperatures the resting fry do not remain viable for very long.

Corrective measures can be take in the event of peat that has dried out too much. For peat in packets, the best is to add more peat of the proper dampness, and the packet sealed. For peat in petri dishes, the dishes can be misted with a spray bottle and resealed.

[18] MARKOFSKY, J & MATIAS, J.R. (1977) The effects of temperature and seasonal collection on the onset and duration of diapause in embryos of the annual fish *Nothobranchius guentheri*. *J. Exp. Zool.* **202**:49–56

[19] VITEK, J & KADLEC, J. (2003) *Hobby compendium: Killifishes.* Jirí Vitek Publishing House of electronic books, Brno.

4 Hatching and rearing of *N. furzeri* fry

Notho eggs can be identified as being ready to hatch when they are "eyed-up."

Eyed-up eggs exhibit two clearly defined eyes peering out of the eggs. These eyes are ringed by a distinct blue or gold iris. When the resting fry within the egg is exposed to light it can be observed to react by twitching and an increase in heart rate[20].

Eggs that fit the above description can be wet as described below; and the fry will hatch (perhaps requiring some encouragement).

4.1 Hatching eyed-up eggs

Transfer the eyed-up eggs to a shallow tub with a tight fitting lid. Add some peat extract[21], at a temperature of about 16–20°C, to the tub. A combination of 50% peat extract and 50% fresh tap water is used for hatching.

Peat extract is prepared by adding hot water to fresh peat and allowing the peat to soak in the water until it sinks. The water is then collected and stored at room temperature until needed.

Stir the water so all the eggs sink. Sink half an oxygen tablet into the water and seal the tub. 12 hours later inspect the tub for fry. (Wetting the eggs just before leaving the laboratory and inspection in the morning is a convenient protocol.)

Remove hatched fry to another tub of the same water quality. The fry can be removed with a tablespoon or gently sucked up with a pipette. Begin feeding with enriched *Artemia* nauplii.

In the event of unhatched eggs, that appeared ready to hatch but did not, several options exist. The eggs may not be ready to hatch, in which case they can simply by restored on peat. Another option is that the fry was not properly stimulated to hatch or the fry is too weak to hatch out on its own. It is not possible to be sure about which cause it is until after the below methods are employed.

Two methods can be employed to stimulate the hatching of reluctant fry. The first method involves adding a volume of cool (10 to 15°C) fresh water to the tub with the unhatched eggs and waiting. Eggs that are ready to hatch can do so within an hour.

Should this fail the eggs can be transferred to a 15 mL plastic screw-cap tube with a small volume of water (3–5 mL). CO_2 is injected into the tube by simply exhaling into the tube a few times[14]. The tube is swirled mildly and inserted into one's pocket whereupon one can continue with other tasks, stopping every 15 or so minutes to inspect the tube. The combination of agitation and increased CO_2 stimulate reluctant fry to hatch[19]. This technique can be very useful in rescuing egg-bound fry of old eggs.

An elevation in partial pressure by sinking the eggs in a jar with loose fitting (but water tight) lid into a deep body of water can stimulate hatching as well as reduce the incidence of belly-sliding[16]. The application of a heavy rock or other object on top of the lid of the hatching container can accomplish the same effect.

Eggs incubated at 27°C and above may require such special encouragement to hatch.

Eggs that have been hatched prematurely will exhibit a clear pale yellow bulge under their bellies with many red capillaries which is the yolk sack. These fry may have trouble

[20] Scheel, J. (1975) *Rivulins of the old world*. TFH Publ. NJ USA.

[21] DE TORRES, J.S. (1997) Las Grandes Cynolebias, Los Killis Del Diabolo. *Boletìn Informativo de la Sociedad Española de Killis*, **74**:37–45

swimming, and seem to skip over the substrate. This is termed belly-sliding. In the event of belly-sliding, the fry can be enclosed in an air tight tub with an oxygen tablet and peat extract. Fry from old eggs may also belly-slide but will lack the yolk sack. These fry may recover in an environment with elevated [O_2] but such fry generally do not survive to adulthood and are very fragile. The simple application of peat extract has also been observed to remedy belly-sliding[22].

Eggs should be hatched in a room with an ambient temperature of no less than 20°C as at cooler temperatures the fry may survive even if they hatch properly. A room temperature of 25°C is fine for the fry to prosper.

A note of caution: it has been observed[23] that peat extract can have a high quantity of free ammonium. In the presence of elevated levels of O_2, the ammonium could be converted to NO_2^- that may kill the fry.

4.2 Caring for newly-hatched fry

It is important to keep the fry above 15°C to ensure survival. Newly hatched fry are sensitive to low temperatures and extreme changes thereof. Organic pollutants are also not tolerated very well.

After hatching, the fry should have been transferred to a new tub with water of the same quality as for hatching; and feeding begun. The author prefers to use 2L ice-cream tubs and fills them to a quarter its volume with hatching mix (as described in the previous section).

The fry are maintained in this volume for 24 hours with twice daily feedings of enough *Artemia* nauplii as what can be consumed between feedings. All uneaten and dead nauplii should be removed from the hatching tub. After 24 hours the tub water is diluted 50% by the addition of ≈ 500 mL of fresh water—bringing the tub volume to ≈50% capacity. After 48 hours the volume is brought up to 75% and after ≈62% hours the tub is filled to capacity. After ≈86% hours the fry are given a 50% water change.

In the mean time a rearing tank is prepared with fresh water. The goal is to have 1 fish per 1–2 L. The greater the volume to fish ratio, the better the growth rate and level of maintenance/disturbance needed to rear the fry. The temperature must be 25°C, and have soft filtration and no strong current. A filter may not strictly be needed if the fry are not very great in number and the tank of suitable size. As long as daily water changes are performed and uneaten food removed the fry will grow properly.

The author will generally float the fry tub in a tank which is at 25°C. If this tank is void of fish the water from it would be used for the water changes. Where possible the would-be rearing tank is the tank the tub is floated in.

After a 5–7 day period with twice daily feedings and 50% water changes the fry can be released into the rearing tank.

The addition of some ramshorn snails (*Planorbis* species) to the rearing tub and tank are of great value in clearing away uneaton food. Care must be taken to check on the state of the snails. A dead snail can rapidly foul a fry tub. A clump of Java moss will help keep the water quality more stable and the tub free of dangerous ammonia/ammonium.

[22] SMITH, C. (2003) Don't throw away that "peat tea." *J. Amer. Killifish Assoc.* **36**(3):105–106

[23] HARRISON, C., pers comm, Killietalk

4.3 Fry rearing from week two

In the second week feeding with enriched *Artemia* nauplii can continue. From the middle of the second week to beginning of the third week feeding with finely chopped *Chironomus* larvae should begin. (A frozen block of *Chironomus* larvae is amenable to being chopped into tiny pieces for feeding.) The chopped *Chironomus* larvae should be fed in the morning in a position where the fry congregate for food (*Artemia* nauplii will migrate to areas of the tank with the best light and the fry will follow). Some fry may begin to pick at this new food.

All uneaten food must be removed from the tank.

Continue with feeding both enriched *Artemia* nauplii and the finely chopped *Chironomus* larvae until all the fry can consume the chopped *Chironomus* larvae, in which case feeding with nauplii can cease.

Feeding with chopped *Chironomus* larvae may have to continue into the fifth week, but ideally by the beginning of the fourth week the fry are consuming whole *Chironomus* larvae.

The fry may need to be sorted by size to avoid cannibalism or any of the smaller fry being abused too seriously by larger tank mates. If the number of fry is too great (> 30 in a 30 L tank) the fish should be split up over more tanks according to size to facilitate proper growth and development.

Ill or stunted fry may grow slower, be less fit and more prone to die for no visible reason. Whether the fry are damaged as fry or eggs is impossible to tell. Elevated levels of ammonia or a strong current during early life could be a cause. Such stunted fry may require enriched *Artemia* nauplii until their fifth week!

After four weeks the fish should be at least between 2 and 2.5 cm in length. Females are normally smaller than males and may only measure 1.5 to 2 cm. Under optimal conditions males can attain 4 cm TL at four weeks and 8 cm TL after eight weeks.

4.4 Accommodating the needs of fry of different species

The fry of *N. furzeri* and *orthonotus* can take enriched *Artemia* nauplii from hatching while the fry of other species may only be capable of taking freshly hatched *Artemia* nauplii. Some species, such as *Fundulosoma thierryi* and *N. rachovii* may require supplementation with vinegar eels or microworms but this has not been the author's (TG) experience.

The newly hatched fry are ravenous and can be easily observed under the microscope to see if they are feeding. Their bellies should be bright orange with the ingested *Artemia* nauplii.

The above care protocol may need to be modified ad hoc for each species. After four weeks, fry of *N. rachovii* Black may still require enriched *Artemia* nauplii and only be beginning to accept chopped *Chironomus* larvae as food.

4.5 Diseases afflicting fry and subadult fish

Notho fry can be very sensitive to velvet disease (parasitation by *Oodinium* sp.). Velvet outbreaks are the result of poor maintenance and the first course of action is a general clean-up of the tank. To treat the infection salt should be added to a concentration of about 3.4 g/L over the course of three days.

Other treatments are acriflavine at 5–10 mg/L. Acriflavine can be purchased at petstores where it is included in various propriety bands at various concentrations. It is best to purchase a brand targeted specifically at velvet or other protozoan parasitic infections and follow the manufacture's instructions.

Caution must be taken with using medications including copper as *Nothos* can be sensitive to copper.

Velvet does tremendous damage to the gills of fish. For this reason the addition of some methylene blue may be of benefit as it aids in respiration.

Notho fry are generally disease resistant except in the case of the above parasite.

A watch should be kept for intestinal worms; and new introductions to the fishroom should be quarantined to prevent the spread of *Glugea*.

Flubendazole can be used to treat intestinal worms at a dose of 2 mg/L. Flubendazole is generally supplied as a 5% powder meaning that 40 mg of the powder would need to be administered to an infected tank. Adding the drug to the fish food (see Section 5.1, page 14) may be more efficient. For the similar compound piperazine, 25 mg per 10 g of food is suggested[24]. This is fed for seven days.

1 ml of a 5% Levamisole solution to every 3.8 L is also effective for intestinal worms[25]. A large water change is performed 24 hrs later. The treatment is repeated 2–4 weeks later.

[24] AXELROD, H.R. (1977) *Diseases of tropical fishes.* TFH Publishing, New Jersey.

[25] Coletti, T (Feb 2005) The Livebearer: New Year's resolution: De-Worm Your Fish! *FAMA* p52–56

5 Experimental protocols

5.1 Feeding protocols

5.1.1 Adding hydrophobic compounds to frozen *Chironomus* larvae

1. Thaw enough *Chironomus* for as many feedings as needed. Allow to drip dry.
2. Aliquot thawed *Chironomus* into ice-cube tray wells, enough for a single feeding.
3. Add compound according to final concentration needed for a single feeding of fish (e.g. 100 μL of 10 mg/mL X solution for 0.1 mg per fish where there are 10 fish). Mix and store in fridge while gelatine is prepared.
4. Prepare 5% gelatine, allow to cool till just above gel point. Add to food, mix and freeze.

Feed fish as per normal.

5.1.2 Adding hydrophilic compounds to frozen *Chironomus* larvae

Provisional protocol. . .

1. Thaw enough *Chironomus* for as many feedings as needed. Allow to drip dry.
2. Aliquot thawed *Chironomus* into ice-cube tray wells, enough for a single feeding.
3. Add compound according to final concentration needed for a single feeding of fish (e.g. 100 μL of 10 mg/mL X solution for 0.1 mg per fish where there are 10 fish). Mix and store in fridge while gelatine is prepared.
4. Prepare 5% gelatine, allow to cool till just above gel point. Add to food, mix and freeze.

Feed fish as per normal. Pray it works!