Video Article

Protocol for Acute and Chronic Ecotoxicity Testing of the Turquoise Killifish *Nothobranchius furzeri*

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Abstract

The killifish *Nothobranchius furzeri* is an emerging model organism in the field of ecotoxicology and its applicability in acute and chronic ecotoxicity testing has been demonstrated. Overall, the sensitivity of the species to toxic compounds is in the range with, or higher than, that of other model species.

This work describes protocols for acute, chronic, and multigenerational bioassays of single and combined stressor effects on *N. furzeri*. Due to its short maturation time and life-cycle, this vertebrate model allows the study of endpoints such as maturation time and fecundity within four months. Transgenerational full life-cycle exposure trials can be performed in as little as 8 months. Since this species produces eggs that are drought-resistant and remain viable for years, the on-site culture of the species is not needed but individuals can be recruited when required. The protocols are designed to measure life-history traits (mortality, growth, fecundity, weight) and critical thermal maximum.

Video Link

The video component of this article can be found at https://www.jove.com/video/57308/

Introduction

Sensitivity profiles of an array of species to strategically selected toxicants have been described[1] for the European REACH legislation (Registration, Evaluation, Authorization, and Restriction of Chemicals). Acute or short-term toxicity tests were mostly used for this purpose as they give a quick indication of a species' sensitivity. However, in their natural environment, organisms are exposed over much longer periods and full life-cycles or even several generations could be affected[2]. Moreover, organisms in polluted environments are typically exposed to more than one stressor at a time, which may interact with each other, possibly resulting in synergistic effects[3]. Hence, safe concentrations calculated based on acute, single stressor toxicity tests may underestimate the actual risks imposed by toxicants in natural environments. It is, therefore, advisable to also study the chronic and multigenerational effects of sublethal concentrations of toxicants in an environmentally relevant context as advocated by the European Commission[4,5] and the USEPA (United States Environmental Protection Agency)[6,7]. Especially in vertebrate research, the costs in terms of labor, money, and time are high when performing chronic and multigenerational exposure studies because of the relatively long lifespan of vertebrates compared to invertebrate model organisms. Therefore, it is advisable to choose the most appropriate fish model organism, depending on the research question. Furthermore, a wide array of vertebrate species should be available in order to test the generality of responses across species to be able to adapt regulations based on the most sensitive species. For now, there is a need to develop new, efficient protocols with vertebrate model species characterized by short life-cycles to lower the costs of performing chronic and multigenerational exposures on vertebrates[7,8].

The turquoise killifish *Nothobranchius furzeri* is an interesting fish model to use in such long-term exposure experiments because of its short maturation time and life-cycle (generation time less than 4 weeks[9]). This means that ecologically relevant endpoints such as maturation time and fecundity can be studied within a short time frame compared to other fish models[7]. Furthermore, these fish produce drought-resistant, dormant eggs that remain viable for several years when stored under standard conditions, thereby eliminating the need for a continuous culture[9]. In ecotoxicological studies, this also implies that replicate fish can all be hatched at the exact same moment, resulting in time synchrony for all animals, even among batches of eggs produced at different times. We advise using the laboratory GRZ strain to perform exposure experiments. This strain performs well under laboratory conditions, is homozygous (except for sex chromosomes) and the genome is well characterized[10,11].

In ecotoxicological studies, it is important to select the appropriate range of test concentrations. Several complementary methods can be used to this end. The nominal concentration range can be based on the sensitivity of a related species, such as *Nothobranchius guentheri*[12]. Alternatively, the range can be based on the sensitivity of standard fish models, such as zebrafish (*Danio rerio*)[2] that have a comparable sensitivity to most toxicants (Philippe *et al.* (in review)). In combination, with both of these options, a range finding experiment should be

conducted to select the nominal concentration range. For acute testing, researchers should aim for concentration treatments with 100% mortality, intermediate mortality and 0% mortality after 24 h of exposure to the toxicant. For chronic testing, it is advisable to run the range finding experiment for two weeks to verify if larval mortality in the condition with the highest test concentrations does not exceed 10% during this reference period.

The protocol can serve as a baseline to perform acute and chronic exposure to waterborne pollutants on *N. furzeri*, examining potential effects of stressors both at the individual and cellular level. It can also be used to perform multi-stressor research to accommodate a higher ecological relevance, mixing different toxic compounds or studying interactive effects between pollution and other natural stressors (*e.g.* predation) or anthropogenic stressors (*e.g.* warming due to climate change).

Protocol

All methods described here have been approved by the Ethical committee of KULeuven.

1. Hatching and General Maintenance of *N. furzeri*

1. Prepare fish medium (pH 7) at a temperature of 14 °C and add purified Type II water, with added standardized salts, to a conductivity of 600 µS/cm (24 °C).
2. Select eggs from the GRZ (Gona-Rhe-Zhou) laboratory line that have been stored under standardized conditions[13]. Select eggs in the DIII stage (*i.e.* ready to hatch), recognizable by the presence of golden eyes[9] and gently transfer them with a soft pair of tweezers to a plastic 2 L tank (no more than approximately 30 eggs per tank).
   NOTE: In order to have a sufficient number of healthy test organisms, hatch twice as many eggs as the number of required fish larvae.
3. Add 1 cm of the fish medium at 12 °C and let the water temperature gradually converge to room temperature (24 °C)[9]. Fish will hatch within the first 12 h.
4. After 24 h, feed the hatchlings a concentrated dose of freshly hatched *Artemianauplii* (for more detail on the frequency and amount of food, refer to the breeding protocol of Polačik *et al.* 2016[9]) and increase the water depth to 5 cm by adding the fish medium.
5. After 36 h, feed the hatchlings another concentrated dose of freshly hatched *Artemia nauplii* and add fish medium to increase the water depth to 10 cm.
6. Incubate fish containers under constant temperature conditions (*e.g.* in an incubator, climate room or heated water bath) under a 14 h:10 h light:dark regime.
7. Before the start of the experiment, complex fish containers (without fish) with the exposure compound by filling them with the highest concentration of exposure medium and leaving it overnight in order to limit the transfer of toxicants to the container in the actual experiment.
8. 48 h after hatching, select healthy buoyant larvae to start the exposure experiment. Discard so-called belly-sliders that were unable to fill their swim bladder and consequently have an impaired buoyancy (continuously sink to the bottom).

2. Short-term Exposure Protocol

NOTE: Researchers should aim for at least 20 replicates (20 fish in separate jars) per treatment. In addition to a full control treatment, a solvent control should be included if the stock solution of the compound is prepared using a solvent. The solvent control should contain the amount of solvent equaling the solvent concentration in the highest exposure concentration.

1. Prepare the experimental containers (0.5 L glass jars) by labeling them and filling them with the appropriate exposure medium (different toxicant concentrations). Add the compound to obtain the correct concentration.
2. Transfer larvae (48 h post-hatching) individually to the containers (1 fish per container for individual monitoring).
   NOTE: Fishes are exposed individually to minimize potential confounding effects of social interaction such as competition for food and aggression. However, fish are allowed to visually interact in accordance to ethical standards for laboratory animal use.
3. Follow up this acute exposure for a duration of up to 2 weeks. During that time, feed the fish *ad libitum* with *Artemia* nauplii twice per day, 7 days/week.
4. Refresh the medium every other day to maintain water quality and to minimize potential effects of compound degradation. Monitor key water variables (dissolved oxygen levels should exceed 80%, conductivity should range between 600 and 700 µS/cm, pH between 7.8 and 8.2, and hardness (as $CaCO_3$) between 350 and 450 mg/L, which lies within the range of optimal rearing conditions for *N. furzeri* [9]). Take water samples before and after refreshing the medium to determine actual compound concentrations.
5. Endpoints
   1. Check fish for mortality, stress (*e.g.* aberrant behavior: swimming upside down) or sickness daily (morning, evening). Consult the publication of Shedd *et al.* (1999)[12] for details on the observation of mortality, stress or sickness.
   2. Calculate LC_{50} values based on mortality using dose-response curves (Ritz and Streibig, 2005) at different time points. Use the *drm* function in the drc package in R v3.2.3 (R Development Core Team, 2016) or similar statistical approaches.

3. Chronic Exposure Protocol

NOTE: Aim at a minimum of 25 fish/condition at the onset of the experiment, to minimize chances of a skewed sex-ratio and to accommodate potential background mortality due to natural causes (*i.e.* age-related mortality).

1. Hatching (see section 1)
2. Phase I (2 days post-hatching - 16 days post-hatching)
   1. Follow protocol as described in 2.1 - 2.4

2. As during the second phase of the experiment, the medium will degrade throughout the week (no refreshment, see below). Store the required amount of medium for a week in large inert containers in order to allow similar degradation of the compound.

3. Phase II (16 days post-hatching - end)
    1. Prepare 2 L experimental glass jars by complexing them with the compound. Fill the jars with the correct exposure medium and add an air tube to aerate the jar. House fish individually in these jars for the remainder of the experiment. Allow visual interaction to accommodate ethical standards.
    2. Refresh the medium once per week. Transfer the fish with a net to a new jar containing the same exposure medium. Take water samples every day throughout a week to monitor the degradation of the compound in each concentration treatment. Calculate a degradation curve for each treatment if multiple stressors are tested (*e.g.* toxicity of a compound in different temperature regimes). Measure abiotic parameters (pH, temperature, % dissolved oxygen, conductivity) three times per week.
    3. From 2 days post-hatching (dph) until 23 dph, feed the fish twice per day, 7 days per week *ad libitum Artemia* nauplii. From 24 dph - 37 dph, complement the *ad libitumArtemia* diet with chopped *Chironomus* larvae. From 38 dph on, feed the fish twice per day, 7 days per week *ad libitum* frozen *Chironomus* larvae.

4. Endpoints
    1. Daily check fish for mortality, stress or sickness[12].
    2. To determine growth, measure the body size on a weekly basis (9 dph - 16 dph - 21 dph - …) by transferring fish to a petri dish filled with medium from their reservoir. Take 4 - 5 size calibrated pictures of the fish from above (at a fixed height) and analyze them digitally using a spatial measuring program (*e.g.* ImageJ).
    NOTE: For adult fish, use a higher petri dish to minimize handling stress by keeping all fish submerged during the whole measuring process.
    3. For male maturation, visually inspect fish daily for coloration from 15 dph onwards. Check the fins for first signs of nuptial coloration (secondary sexual characteristic). Use the first day at which this is visible as a proxy for male maturation time.
    4. Couple non-sexed fish with males of the same treatment group or non-experimental males three times per week from 30 dph onwards in order to determine female maturation time (the day the first egg is deposited). For this, use the spawning protocol described in 3.4.5.
    5. For fecundity, couple mature females with mature males 3 times/week from 30 dph onwards, within their treatments using a crossing scheme.
        1. Prepare a spawning tank (1 L) for each couple, using exposure medium from the male aquarium supplemented with spawning substrate (fine sand <500 µm).
        2. Transfer both the male and female into the spawning tank and allow them to spawn for two hours. Minimize human activity or disturbance around the spawning containers during this process.
        3. Afterwards, gently transfer fish back to their original housing containers, without unnecessary mixing of the water, which would whirl up the eggs in the spawning substrate.
        4. Filter the eggs out by pouring the spawning substrate over a 500 µm mesh. Count the eggs and transfer them (using soft tweezers) to damp peat moss in Petri dishes[9,14].
        5. Remove dead eggs daily. After a week, seal the Petri dish with sealing film and store it in a temperature controlled incubator at 28 °C and a 14:10 h light:dark cycle for immediate development to the DIII phase (*i.e.* ready to hatch after approximately three weeks). For long-term storage, store the eggs at 17 °C in constant darkness upon which eggs enter the dormant phase and remain viable for multiple years. When recruiting fish from these dormant eggs for experiments, transfer the dormant eggs to 28 °C conditions with 14:10 h light:dark cycle for approximately three weeks to allow for development to the DIII phase.

    6. Measure the Critical thermal maximum (CTmax) (a measure for performance[15]) of adult fish.
        1. Use a water bath that is heated at a constant rate of 0.33 °C/min and in which the water is continuously circulated. Add several 1 L aquaria for each individual fish.
        NOTE: Given space constraints in the water bath, it is necessary to work in several series. Potential differences in conditions among series should be taken into account when performing statistical analysis by including 'series' as a random factor.
        2. Start the trial by adding the fish to the aquarium when the water in the aquarium has reached the experimental rearing temperature of the fish (generally 28 °C). Monitor the temperature in the 1 L aquaria of the CTmax bath every 5-min using a digital thermometer (0.1 °C scale).
        3. End the trial when the fish fails to maintain a dorso-ventrally upright position or starts twitching heavily[16,17]. Measure the temperature in the 1 L aquarium, which is the critical maximal temperature. Transfer the fish back to its experimental housing for recovery.

    7. Measure the weight (0.1 mg accuracy) of the fish on the last day of the experiment by patting it dry and transferring it on a weighing boat. NOTE: All fish should be measured four hours after the last feeding to standardize food weight in the intestinal tract.
    8. Euthanize the fish using 0.1% Tricaine.

4. Transgenerational exposure protocol

NOTE: To measure transgenerational effects of pollutants on *N. furzeri*, follow the chronic exposure protocol outlined above for the first generation.

1. Twice weekly, check the development of the produced eggs (i.e. the second generation) stored at 28 °C 14:10h light:dark cycle conditions by inspecting the petri dishes for embryos in the DIII phase (see Polačik *et al.* 2016[9]). When more than 50 replicates of each parental treatment are fully developed, hatch them following the protocol in 1.1.
2. Expose healthy, buoyant fish to exactly the same set-up and treatment as the parental fish.

Representative Results

The results of the acute exposure of *N. furzeri* to different concentrations of copper, calculated as in 2.5.2, show cleardose-response relationships (**Figure 1**). There is an increase in mortality with increasing toxicant concentration. LC_{50} values decrease over time, meaning that with decreasing concentrations, more time passes before 50% of the replicates die. For detailed results on the acute and chronic exposure of *N. furzeri* to copper, as well as the comparison of the species' sensitivity compared to other species, we refer to Philippe *et al.* 2017[7].

In the chronic exposure trial, body size and fecundity are sensitive endpoints. There can be extensive variation in the growth of the fish, depending on the temperature[18]. Adult sizes between 30 and 50 mm are considered normal in this set-up. The set-up allow finding differences between treatments (**Figure 2A**). In a chronic exposure assay using waterborne copper, there was a significant effect of copper exposure during week three ($\chi^2_{5,34}$ = 40.7, *P* <0.001), with *N. furzeri* exposed to 19.38 µg/L Cu being smaller than all other fish (all *P* <0.001) (**Figure 2B**). For fecundity, control values should fluctuate between around 50 eggs per week per female at the peak of egg production[7]. In another experiment, using waterborne chlorpyrifos and a 2 °C temperature rise, we found a significant interaction between temperature rise and chlorpyrifos exposure on fecundity ($\chi^2_{2,202}$ = 25.3, *P* <0.001). At 28 °C, fish exposed to 4 µg/L produced less eggs compared to fish exposed to 2 µg/L and control fish (both *P* <0.001) (**Figure 2B**). At 30 °C, control fish produced more eggs compared to all chlorpyrifos exposed fish (both *P* <0.007). Both the main effect of exposure to chlorpyrifos ($\chi^2_{2,202}$ = 96.8, *P* <0.001) and the main effect of temperature ($\chi^2_{1,202}$ = 10.18, *P* <0.001) significantly reduced fecundity. The measurement is quite time consuming because of the handling of the fish and the plating of the eggs on peat[9], but it is often the most sensitive endpoint.

Maturation time is most often affected by pollutants in males. Male maturation time was significantly affected by chronic exposure to waterborne chlorpyrifos ($\chi^2_{2,41}$ = 11.79, *P* = 0.003), with males exposed to 4 µg/L CPF (C2) having an 18% slower maturation compared to control males (**Figure 3A**). This response should, however, be interpreted with caution since maturation time is scored indirectly by determining the onset of nuptial coloration as a proxy. Although males are considered to mature a few days after the appearance of coloration[9], there may be some error on the exact timing of maturation using this measure.

Near the thermal maximum, fish exhibit erratic swimming, increased opercular movement and loss of ability to remain in a dorso-ventrally upright position [16,17]. CTmax values differ between *N. furzeri* strains. Natural populations have CTmax values between 39 °C and 42 °C when reared in temperatures between 24 and 28 °C (**Figure 3B**). The inbred GRZ strain, however, already reaches its thermal maximum at around 37 - 38 °C, even when reared at 28 °C. Whereas this procedure is not lethal to the fish, rare cases of mortality do occur. Such fish are best excluded from the CTmax analysis, as they most likely represent fish that are in relatively poor overall condition.

Previous results mostly showed that CTmax can be affected by the pollutant, in this case, 3,4-DCA ($\chi^2_{2,71}$=17.65, P <0.001) with fish exposed to 0.1 mg/L 3,4-DCA having a 0.32 °C lower thermal maximum compared to control fish (P <0.001). Also, CTmax was affected by the rearing temperature ($\chi^2_{1,71}$=322.0, P <0.001) and fish that were reared at 28 °C had a 1.3 °C higher CTmax compared to fish that were reared at 24 °C.

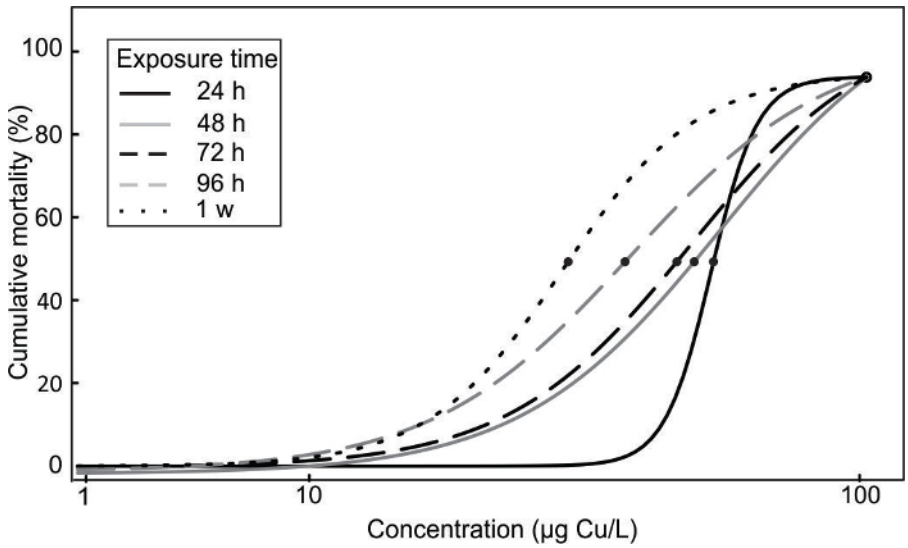

Figure 1: Dose-response curve for copper exposure. Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of the copper exposure concentration (in µg/L Cu and in relation to exposure time). Dots indicate LC_{50} values. This figure has been modified from Philippe *et al.* 2017[2]. Please click here to view a larger version of this figure.

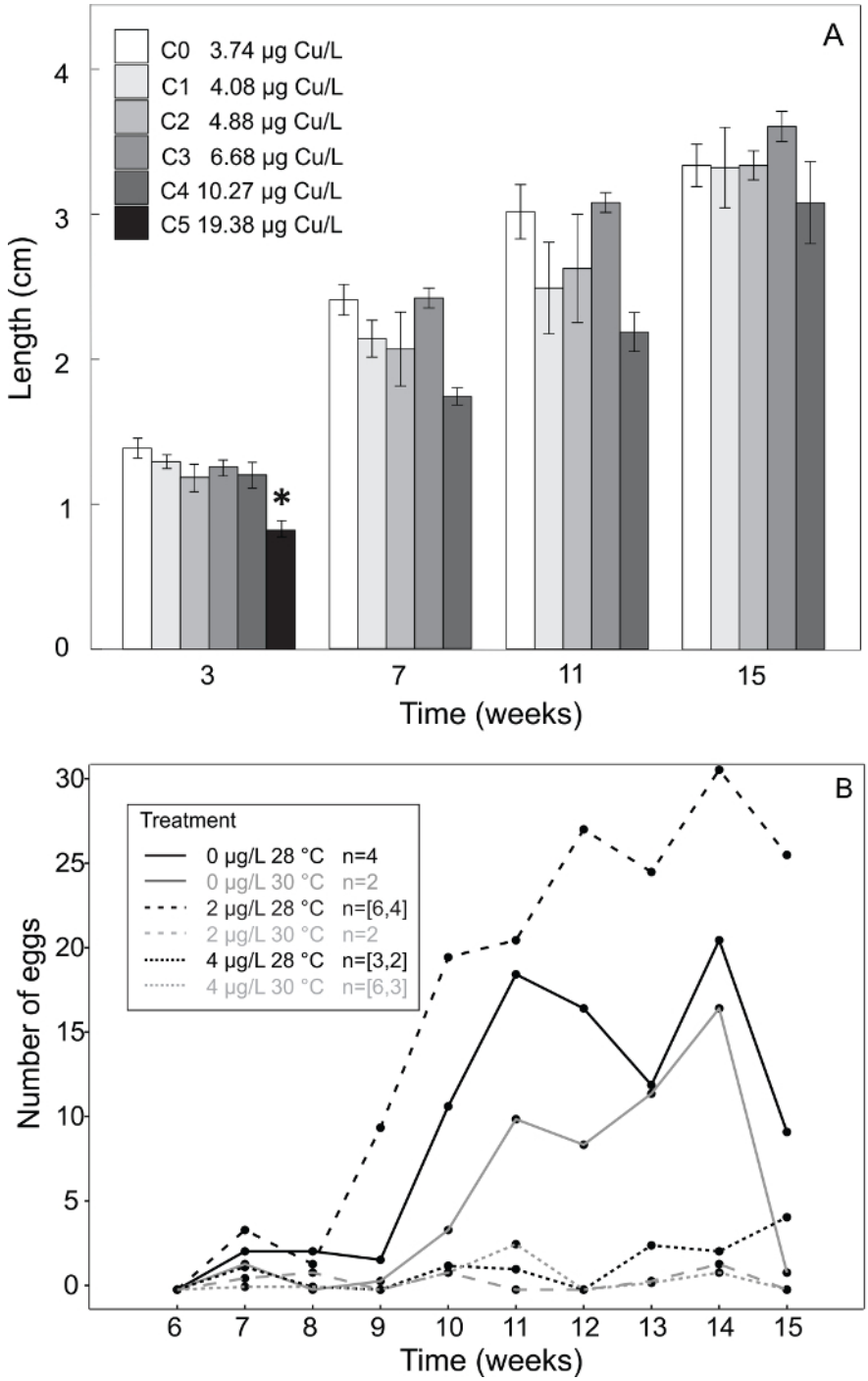

Figure 2: Effects on size and fecundity as endpoints. A) Size (in cm) of *Nothobranchius furzeri* exposed to different concentrations of copper at week 3, 7, 11, and 15. Asterisk indicates that C5 fish are smaller after three weeks at the significance level of P <0.05. Values are presented as mean ± SEM. Sample sizes are n = 6; 6; 7; 7; 7; 7 in week 3, n = 6; 6; 7; 7; 4 in week 7, n = 5; 4; 5; 5; 3 in week 11 and n = 5; 3; 3; 5; 2 in week 15. **B)** Fecundity through the time of fish exposed to different concentrations of chlorpyrifos, crossed with two temperature treatments, measured as the number of eggs per week. To improve the readability and interpretability of the figure, error bars are not shown on the graphs. The number of females in each treatment at the beginning and end of the egg laying period is indicated using the letter 'n'. Please click here to view a larger version of this figure.

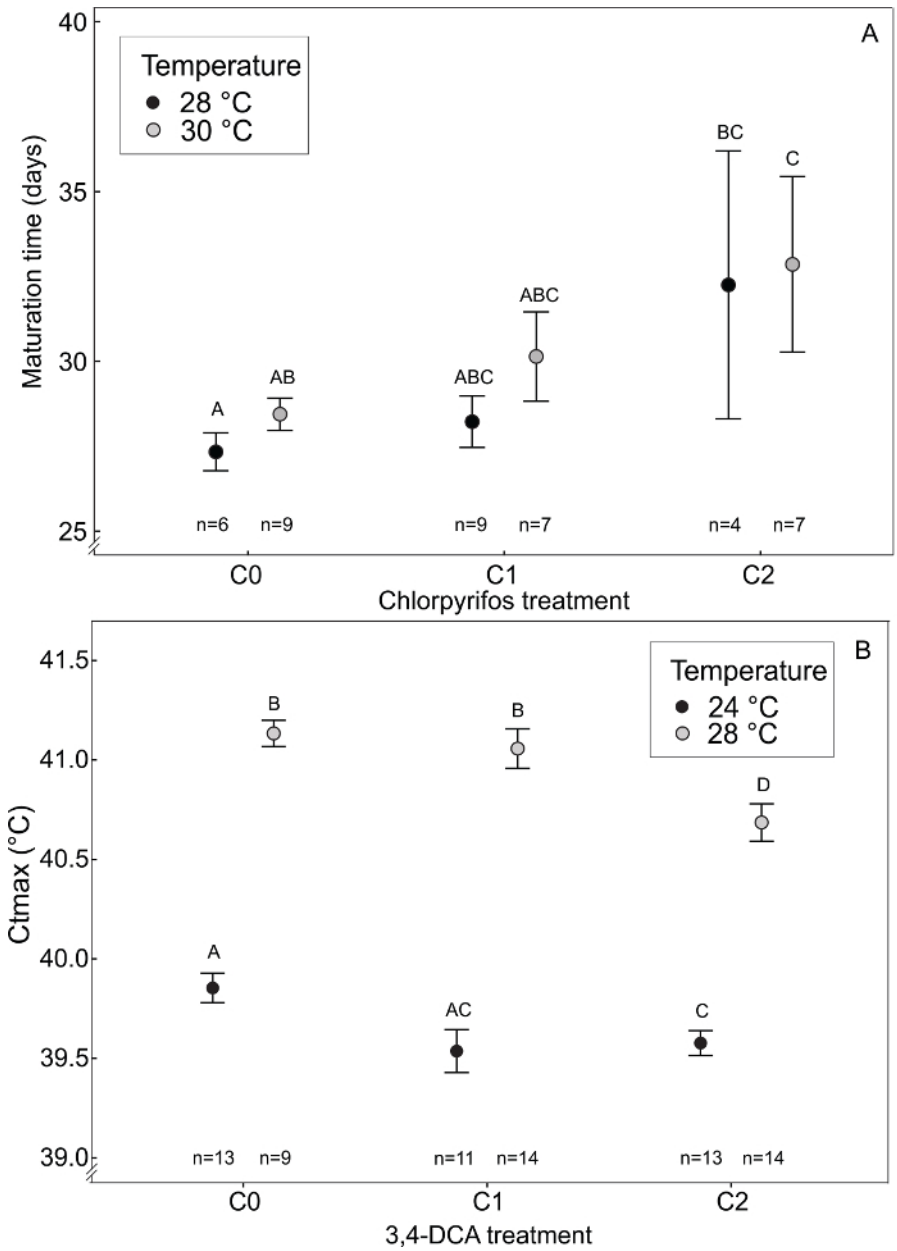

Figure 3: Effects on maturation time and CTmax. A) Mean age (in days) at which the first signs of coloration appeared in males of *Nothobranchius furzeri* exposed to different chlorpyrifos concentrations (0 µg/L (C0), 2 µg/L (C1) and 4 µg/L CPF (C2)) and two temperatures (28 °C and 30 °C). **B)** Mean critical thermal maximum (CTmax) of fish exposed to different concentrations of 3,4-DCA (0 mg/L 3,4-DCA (C0), 0.05 mg/L 3,4-DCA (C1), and 0.1 mg/L 3,4-DCA (C2)) and two temperatures (24 °C and 28 °C). Nominal concentrations are presented as mean value ± SE. Please click here to view a larger version of this figure.

Discussion

This work describes a new bioassay using *Nothobranchius furzeri*, an emerging model organism, to study the individual and combined long-term effects of toxicants and other stressors. The presented protocols were successfully applied to measure the sensitivity of the species to an array of toxicants (copper, cadmium, 3,4-dichloroaniline, and chlorpyrifos). Due to its fast life-cycle, this vertebrate model allows for assessment of sublethal and transgenerational effects within four months. Another major advantage of using this fish species as a model for toxicity screening is the fact that it produces drought-resistant eggs. This enables researchers to store eggs or obtain them from a supplier and eliminates the need for a costly and time consuming on-site culture. Moreover, the embryos can be stored for several years until hatchlings are needed[12].

After studying the sensitivity of *N. furzeri* to a number of reference toxicants, we can add that the sensitivity of the species is in range with, or higher than, that of other model species, depending on the tested compound. Measuring effects on fecundity, in particular, can increase the comparability of the sensitivity on the studied species, as it is a routinely measured endpoint in other model species. Finally, the extent to which

multiple stressors exert adverse effects, when administered individually or combined, is dependent on the evaluated endpoint and the exposure concentration.

There are still some limitations when working with *N. furzeri*. One of the most important limitations is the standardization of food. Batches of *Artemia* cysts or bloodworms can differ in quality and can, as such, impact the results of the study. It is therefore advisable to order a large batch of food to use during the whole length of the experiment.

We believe that this protocol is widely applicable for ecotoxicological screening. *N. furzeri* is rapidly developing into a standard test species in ecotoxicology. The availability of this standard protocol may fuel its establishment.

Disclosures

The authors have nothing to disclose.

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