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Social context and chemical stress: investigating impacts on aging and reproduction in the turquoise killifish *Nothobranchius furzeri*

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Thesis presented to obtain the diploma of Master
in Biology of the Organisms and Ecology

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Academic year 2022-2023

Acknowledgments

The realization of this thesis would not have been possible without the help of my thesis supervisor, Frédéric Silvestre, who gave me precious advice and feedback on the progress of my work.

I would also like to thank Antoine Wittorski for having supervised me and for sharing his knowledge on N. furzeri. The setting up of the breeding would have been much more complicated without his explanations.

Thanks to my teammate, Gaël Fassiau, for sharing with me this unique and challenging experience. We finally did it!

Thanks also to all the LEAP team for their help and their thoughts during the lab meetings. And more particularly, I would like to thank Anthony Mathiron for his help and advice in order to improve more and more this work in terms of statistical analysis and writing.

The great team of students that we were made this experience much more enjoyable. Thanks to Amandine Letexier, Antoine Bouchat, Cassandra Liévin, Charlotte Wouters, Lisa Guerriero and Océane Vermeulen for their support in the moments of questioning and their great team spirit.

Finally, a huge thank you to my parents for their support during this important year (and all the others) and to my sister for checking the English language of my thesis.

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Abstract

Senescence affects the entire biological processes of living beings. It is a difficult concept to study *in vivo* because it involves a longitudinal analysis across the entire life of organisms. Recently, *Nothobranchius furzeri*, the turquoise killifish, has been put forward as a model species for the study of aging. Its rapid life cycle adapted to its ephemeral natural environment results in a very short lifespan of four to six months. In this study, we explored the interaction between environmental factors, such as social context and chemical stress, with senescence and reproductive course in *Nothobranchius furzeri*. First, we investigated effects of the environment on the mortality and survival. Early exposure (0 to 7 days post hatching) to 100 μg/L of the neurotoxicant pesticide permethrin did not affect the survival of individuals. However, living in a community has a negative impact on the long-term survival of fish, which died on average 50 days earlier than individuals isolated from any social contact since their hatching. Second, we investigated the effect of the environment on reproduction. We evidenced that an early exposure to permethrin causes delayed effects on the reproduction of *N. furzeri*. At their 18th week post hatching, exposed individuals laid on average half as many eggs. Moreover, this experiment showed an effect of age on the reproductive capacities of this species. This study highlights the value of longitudinal measurements in defining multiple and delayed effects of the environment with aging and life history traits in the turquoise killifish, a short-lived fish.

Key words: *Nothobranchius furzeri* – aging – social context – reproduction – permethrin

A. Introduction

Senescence is defined as the progressive decline of physiological functions that increase the vulnerability of the organism until its death (López-Otín *et al*., 2013; Lang *et al*., 2013). It has often been considered as the process that allows the turnover of generations within a population to reduce competition for limited environmental resources (Guerreiro, 2012; Kirkwood *et al*., 1991). The question of programmed senescence was then raised. But in nature, very few individuals die of old age; most are eliminated as soon as they begin to weaken. This allows generations to follow each other without competing for food and space resources (Lang *et al*., 2013).

Many physiological mechanisms have been studied as proximal causes of senescence. Cells are affected in their functionality by all types of stress (e.g., heat stress, barometric stress, ionizing radiation). In the case of aging, oxidative stress seems to play an important role. The theory of free radical aging (FRTA) was proposed by Denham Harman (1992) who focused on the harmful properties of free radicals, by-products of aerobic respiration essential for energy production (Harman, 1992; Pole *et al*., 2016; Pontzer & McGrosky, 2022). These are able to intervene in senescence-related mechanisms such as the incidence of age-related diseases or aging, which can ultimately impact the lifespan of cells (Pole *et al*., 2016). This theory is based on data showing that a low-calorie diet and the use of inhibitors of oxidative reactions could increase the life expectancy of a human being by at least 5 years. Another theory is the "Wear and Tear Theory", which suggests that the use and the stress on organs leads to their progressive deterioration and dysfunction, causing them to age, much like objects (Sattaur *et al*., 2020). Many other genetic theories, such as the "Telomerase Theory" attempt to explain the phenomenon of senescence (Musnaini *et al*., 2023).

Evolutionary theories have also emerged over the past century. Senescence appears to be one of the least sensitive processes to natural selection, in contrast to others such as growth or reproduction that occur more rapidly over the course of a lifetime (Terzibasi *et al*., 2008; Žák & Reichard, 2021). Different evolutionary theories of aging have been developed to explain the rules that govern this process (Žák & Reichard, 2021). The best known are based on two main ideas: the first stems from the weakening of natural selection after maturity as a result of too many environmental causes of mortality (predation, fighting, etc.), allowing late deleterious mutations to persist in populations (Haldane, Medawar and Williams in the 1940's and 1950's)

(Fabian & Flatt, 2011; Žák & Reichard, 2021). Several studies on *Drosophila melanogaster* allowed the "Mutation Accumulation Theory" to find empirical support in the early 20th century (Fabian & Flatt, 2011). The second describes senescence as the inevitable decline due to early reproductive investment and leads to the "Antagonistic Pleiotropy Theory" and the "Disposable Soma Theory" suggested by Williams (1957) and Kirkwood (1977), respectively (Fabian & Flatt, 2011; Kirkwood, 1977).

The "Antagonistic Pleiotropy Theory" is based on the presence of pleiotropic genes encoding both early positive events and late deleterious events (Kirkwood & Austad, 2000; Terzibasi *et al*., 2008). Senescence would be one of the delayed effects associated with a beneficial effect. Selection is based on this beneficial effect that is increasingly present in the population (Kirkwood & Austad, 2000). Leroi *et al*. (2005) demonstrated the ubiquity of antagonistic pleiotropy genes and the involvement of the Igf1r gene in the control of longevity among others by its interaction with insulin receptors (Leroi *et al*., 2005). A second theory, the "Disposable Soma Theory", explains that energy utilization in reproduction is preferred over error regulation in somatic cells, which leads to the accumulation of genomic damage and would result in senescence (Kirkwood, 1977; Kirkwood & Austad, 2000; Liu & Sabatini, 2020; López-Otín *et al*., 2013). These two theories show the presence of trade-offs between the life processes of individuals.

In the environment, energy is provided in limited quantity by food. Therefore, energy supply is the primary limitation for life history traits such as development, growth, reproduction, feeding, cell maintenance, and survival (Api *et al*., 2018; Cellerino *et al*., 2016; Kim *et al*., 2016; Lawson *et al*., 2022). Reproduction is an important and essential process for the fitness of organisms and can affect the survival of a population. It represents a significant energy cost in the life of an individual (Cellerino *et al*., 2016; Kim *et al*., 2016; Lawson *et al*., 2022; Scott *et al*., 2022). The impact of reproductive costs on lifespan and aging is one of the most studied tradeoffs (Graf *et al*., 2010; Husak & Lailvaux, 2022; Komar *et al*., 2022). Trade-off between current and future reproduction is clear, early reproductive maturity will negatively affect subsequent reproduction, but evidence for a direct impact of reproductive function on survival and aging is more difficult to demonstrate (Pontzer & McGrosky, 2022).

Furthermore, genetics is not the only drivers of this trade-offs, interactions and social context are parameters of community life that encompass energy-intensive behaviors and functions such as aggressiveness, competition and social stress that interfere with energy

availability (Husak & Lailvaux, 2022). Graf *et al*. (2010) have already shown that these parameters could interfere senescence. In the wild environment, reproduction is an integral part of community life, so it would be essential to include these parameters in order to understand how this lifestyle impacts survival and senescence.

Both reproduction and reproductive senescence are subject to tradeoffs. Changing environmental conditions can influence the balance of the energy budget devoted to this function (Cellerino *et al*., 2016; Kim *et al*., 2016; Komar *et al*., 2022; Lawson *et al*., 2022). For example, the presence of anthropic chemical compounds in the environment can alter the reproductive process of exposed animals. Prabhu *et al*. (2022) showed that toxic molecules such as bisphenol A are able to significantly reduce the reproductive lifespan of female mice. Currently, permethrin known as a neurotoxic insecticide has replaced many compounds (chlorpyrifos, organophosphates) in the pesticide market due to its better photostability, more selective toxicity and high effectiveness (Aznar-Alemany & Eljarrat, 2020; Deepika *et al*., 2022; Goulin, 2016; Tang *et al*., 2018). Despite these advantages, this neurotoxicant attacks the activity of voltage-gated sodium or calcium channels and calcium-ATPase pumps, a neuronal stimulation pathway highly conserved by natural selection between insects and vertebrates (Goulin, 2016; Thoré *et al*., 2020). In addition to its neurotoxicity, hepatic, cardiac, and immune toxicity as well as ROS (Reactive Oxygen Species) production and increased oxidative activity have also been observed (Goulin, 2016; Nunes *et al*., 2019). Estrogenic and anti-androgenic actions of permethrin also impact the reproductive and endocrine systems (Ravula & Yenugu, 2019; Tange *et al*., 2014). In aquatic organisms, the presence of permethrin causes epigenetic deregulation and a decrease in the number of eggs laid by females, resulting from its endocrine-disrupting properties (Blanc *et al*., 2020). This shows that reproduction is also subject to compromise and regulation by external factors. However, while many studies inform on the direct reproductive toxicity of an environmental chemical on aquatic organisms, the question of the effect of such early permethrin exposure on the entire reproductive life of individuals remains unanswered.

If senescence is an ubiquitous natural process that begins during embryogenesis , it can be more or less rapid depending on the species, which determines their lifespan (Pole *et al*., 2016; Žák & Reichard, 2021). The study of this process requires an analysis on the whole lifetime. This is very time consuming and complex in most mammals, which often show little sign of aging and whose lifespan is counted in years. Fortunately, a small teleost in the

cyprinodont clade offers a unique advantage by allowing longitudinal analysis at the individual level of senescence (Cellerino *et al*., 2016; Hellou, 2011; Nunes *et al*., 2019; Thoré *et al*., 2020; Žák & Reichard, 2021). *Nothobranchius furzeri* is found in Southeast Africa in a highly unstable habitat that experiences monsoons (Api *et al*., 2018; Cellerino *et al*., 2016; Žák & Reichard, 2021). This small fish, averaging seven centimeters in length, has acquired a life cycle adapted to the rainy season and lives for a period of four to six months in temporary freshwater (Cellerino *et al*., 2016; Thoré *et al*., 2020) This short lifespan dictates a very rapid life cycle, predicting accelerated senescence (Žák & Reichard, 2021). This very specific characteristic makes this species the shortest-lived vertebrate ever known (Api *et al*., 2018; Cellerino *et al*., 2016). The turquoise killifish is a unique model species for studying senescence, living fast but dying old. Genome sequencing of this specific fish shows that this species goes through the same life stages as other long-lived vertebrates (Hellou, 2011; Thoré *et al*., 2020). The use of *N. furzeri* will allow us to discover how its reproduction is impacted by the social and chemical environment and what are the consequences of these potential effects on age-related processes.

In the present study, we explored how environmental factors (i.e., social contact and permethrin exposure) influenced life history traits and reproductive parameters of *N. furzeri* at different levels: i) the impact of reproduction and social context on lifespan, ii) the impact of chemical stress on lifespan, and iii) the impact of chemical stress on reproduction. Longitudinal data based on repeated measurements throughout the lifespan of individuals were recorded. In addition, the impact of reproductive and social activities on lifespan was studied.

B. Material and methods

1. Laboratory breeding

N. furzeri individuals come from the GRZ lineage that was first collected in Zimbabwe in 1968 and was then maintained in captivity for more than 100 generations (Žák & Reichard, 2021).

1.1. Living conditions

Turquoise killifish were raised in tanks where the physico-chemical parameters of the water were kept constant. The conductivity was 600 μS and the temperature 27 +/- 1°C. To do this, the tank water was composed of reconstituted water, i.e., 30 g of salt InstantOcean® are mixed in 100 liters of distilled water. The temperature was regulated by an Aralab company incubator. Once a week, half of the water from each tank was renewed to keep its parameters constant. Food scraps and dejections were daily removed and replaced with fresh water.

All the individuals were fed *ad libitum* with *Artemia salina* twice a day, (i.e., in the morning and in the afternoon). At their sixth week of life, bloodworms (*Chironomus sp.*) were also added to their diet.

A fixed day:night cycle of 10:14 hours was established in the incubator.

1.2. Breeding

Some eggs were ordered from the MicroBioTests company and a farm was then set up to produce enough eggs for the experiment. Harems of 1 male and 3 females were created at sexual maturity to obtain a high yield without exhausting the females. Spawning trays filled with one centimeter of fine sand was an adequate spawning substrate for *N. furzeri*. The eggs were collected every two days, then sorted and cleaned according to a standardized protocol (App. 1).

The fish laied in two cohorts of 38 and 98 individuals (July 19th and August 9th 2022, respectively). Eggs were ready to hatch after 3 weeks of development when they reached the "golden eyes" stage (Fig. 1; App. 1). To trigger hatching, eggs were transferred into 1 cm of 0.33 g/L humic acid at 4°C (diluted in system water). As collective hatching favored higher

hatching rates, about 50 eggs were placed per tank (Polačik *et al*., 2016). The humic acid tank was slightly tilted to reduce the water level on one side and allow the larvae to fill their swim bladders. After 24 hours, the humic acid was diluted with the same quantity of water from the system. The next day, the larvae were moved to fresh tank water without humic acid.

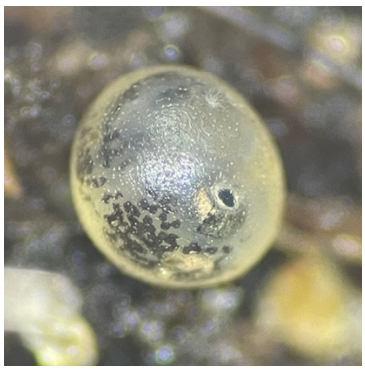

Figure 1: Egg at golden eyes stage.

2. Environmental impact assessment

In order to understand how the environment can impact life-history traits and reproduction of the turquoise killifish, the influence of two factors were investigated: the social context represented by either groups living in community or alone-living individuals, and the presence/absence of the neurotoxic permethrin molecule. One-day-old larvae were randomly assigned to four different groups: i) community group exposed to 0 μg/L of permethrin (control; N = 40), ii) community group exposed to 100 μg/L of permethrin (N = 40), iii) control isolated individuals (N = 27), and iv) isolated fish exposed to 100 μg/L of permethrin (N = 29).

At the beginning of their life, fish from the community groups were kept in community pools of 20 larvae (1.5 L) while each isolated fish was placed in an individual container in a multi-wells plate (well of 10mL per well). Each well was prevented from external visual cues to keep the larvae free from any social contact. As the fish grew, they were placed in larger volumes of water until they reached about 2.5L of water per single adult fish and 40L per community group of 10 fish (5 females and 5 males, this sex-ratio evolved according to the mortality in the groups).

Exposure to permethrin was carried out at the larval stage from one day post hatching (dph) to 7 dph. To do so, working solutions of permethrin at 100 μg/L and at 0 μg/L (control) were prepared from stock solutions at 100 mg/L and 0 mg/L of permethrin in 100% of DMSO,

respectively. Every day of exposure, 200 μL of each stock solution was diluted in 2L of tank water (conductivity: 600 μS, temperature: 27 +/- 1 °C). Final working solutions were obtained in 0.01 % of DMSO. Exposure water in the wells or in the pools were completely renewed every day to ensure that real concentrations were as close as possible to the nominal ones. At the end of exposition, fish were maintained in clean tank water.

2.1. Reproduction analysis

2.1.1. Experimental procedure

Reproductive tests were performed only in the community group. Once the sexual maturity was reached and the characteristic coloration of both sexes was visible, around their 5th week post-hatching (wph), the reproduction could begin. Data on eggs were collected from the 7th wph of parents. At the beginning of this experiment, adult couples were formed and laid eggs were collected at different fixed times of the week. Each week the same manipulations were performed in order to obtain comparable results (Fig. 2).

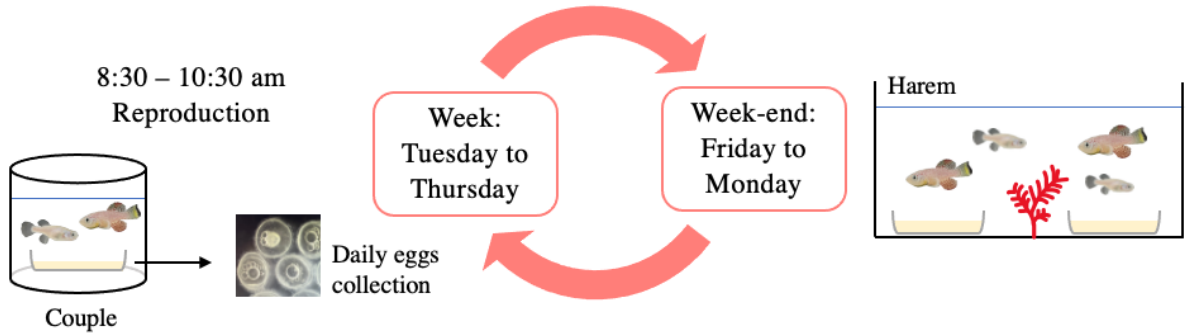

Figure 2: Typical week for the community group. During the week, pairs consisting of one male and one female were formed between fish from different harems that have been exposed to the same permethrin concentration. On Tuesday, Wednesday and Thursday, each pair was placed in a breeding tank (about 2.5 L) with a sandbox for two hours. After this time, around 10:30 am, the two individuals were separated until the next day. The eggs laid during this period were then recovered. This was repeated three times during the week. After the reproduction of Thursday, the couples were all put back in their harem of origin until the next Monday. One sandbox per male was also added to the harems. On Monday morning, the sandboxes were removed to stop the reproduction.

During the weekends, *N. furzeri* were placed in groups of about 10 fish in community aquaria. In their natural environment, this species uses sand at the bottom of temporary puddles as spawning substrate. The males must then defend their territory to attract females. To mimic this behavior, sand trays were added to provide adequate spawning substrate. The number of

these tanks was equal to the number of males in order to provide to each one a territory to defend and to recreate a dynamic present in the natural environment.

On Monday mornings, the sandboxes were removed from the community trays and the breeding event ends. Each male was then separated from the females and placed in a breeding tank (about 2.5L) containing a new sandbox for the rest of the week. Males acclimated alone to their new environment during one day to reduce the impact of stress on the breeding event. The separation of about 24 hours of the two sexes also allowed the females to regenerate their egg stock before the next reproduction.

On Tuesday mornings, at 8:30 am, a female was randomly placed with each male. The two partners were left together until 10:30 am before being separated. The female was identified and placed in another 2.5L tank so that each pair remains identical for the rest of the week. The sandbox was removed from the breeding tank to collect the eggs it contains and then placed back in each tank. This step was done three times during the week, on Tuesday, Wednesday and Thursday mornings.

On Thursdays, after the spawning event, all fish were returned to their respective group with new sandboxes until the following Monday.

Eggs were collected four times each week. The Monday harvest was a reset because it is possible that some females did not undergo a spawning event the week before. It is therefore necessary to eliminate the eggs produced by females during this week before starting a new one. For the other three harvests, the conditions were identical, the reproduction took place during 2 hours each morning and was separated by approximately 24 hours of rest from the previous one. The pairs were also the same throughout the week in order to obtain replicates. Only these data will be analyzed in order to keep conditions that allow comparison between groups.

2.1.2. Reproduction indexes

The sand of each box was sifted at 500 μm separately to extract the eggs. These were then directly sorted with a binocular to count the total number of eggs (laid in the 2 hours of reproduction) that give the fecundity and to remove the dead eggs (App. 1). The indices used in this study were chosen on the basis of Žák & Reichard (2021) which performed the same

type of analysis of reproduction. Here, the fertility index is based on the number of alive eggs laid by the female (Žák & Reichard, 2021):

$$Fertility\ index = \frac{alive\ eggs}{total\ number\ of\ eggs}$$

After this first count, the eggs were cleaned by a bleaching step. They were successively soaked for about 30 seconds in different baths of H_2O_2 (0.5 %), fresh system water and in methylene blue (1 %) (App. 1). They were then kept in a petri dish annotated with methylene blue. The next day, after about 24 hours, the eggs were again observed with a binocular to remove the unfertilized eggs. The eggs were then kept in methylene blue for the rest of the week. The fertilization index is based on the number of fertilized eggs (Žák & Reichard, 2021):

$$Fertilization\ index = \frac{fertilized\ eggs}{alive\ eggs}$$

To initiate embryo development, *N. furzeri* eggs must be both on a substrate and in a humid environment. This compromise was obtained using coconut fiber. This one was wrung out and the excess of water was removed. The coconut fiber was prepared and compacted in petri dishes according to a standardized protocol (App. 1). On Fridays, after the last observation with the binocular, eggs were placed on the coconut fiber. Boxes were sealed with parafilm and placed in an incubator at 28°C. The development of the embryos took between 2 and 4 weeks. Eggs were observed regularly to recover them when they reached the "golden eyes" stage and to measure the last index related to the development (Žák & Reichard, 2021):

$$Development\ index = \frac{eggs\ at\ golden\ eyes\ stage}{eggs\ put\ on\ the\ coconut\ fiber}$$

2.2. Study of senescence

2.2.1. Morphological measurements: length

In order to investigate the evolution of the individuals' growth in the diverse conditions, the total size (from the beginning of the head to the end of the tail) of each fish was established at 1 dph (i.e., 0 wph), 7 dph (i.e., 1 wph), 5 wph, 12 wph and 17 wph. To do so, a picture of the individuals was taken at each time points and further analyzed thanks to the software ImageJ.

A grid placed on the photo was used as a scale. From the 5th week onwards, the fish had to be anesthetized to obtain accurate data; only the males were therefore measured.

2.2.2. Survival

To understand the impact of the environment on *N. furzeri* lifespan, date of hatching and death were recorded for each fish.

2.2.3. Molecular analysis

The effect of parental age was also investigated on eggs at the "golden eye" stage from exposed fish. Three time points were studied to compare eggs laid at the beginning of the reproductive life at 8 wph, in the middle at 12 wph and at the end of the reproductive life at 15 wph. Pools of 2 eggs were formed for each sample in order to have enough genomic material. The number of samples (N) per condition (time points and permethrin concentration) was 8. The aim of these molecular analyses was to show a difference in the expression of the genes of interest in the eggs laid by the exposed parents. For this purpose, the RNA concentration was analyzed by RT-qPCR (Real Time quantitative Polymerase Chain Reaction).

2.2.3.1. RNA extraction

RNA extraction was performed with the Quick-DNA/RNA Miniprep Plus kit (#D7003) from Zymo Research and following an adapted protocol based on that provided with the kit (App. 2). Homogenization of the eggs was performed by grinding zirconium beads and treatment with proteinase K. RNA was eluted in 30 μL of DNase/RNase free water.

The extracted samples were tested with nanodrops to know its RNA concentration and its level of solvent or protein contamination (App. 3). An 1.5 % agarose gel migration was also used to confirm the good realization of the RNA extraction (App. 4).

2.2.3.2. DNase et Reverse Transcription

DNase treatment was applied to ensure that the sample contains only RNA prior to retrotranscription. This step is performed with the DNA-free™ DNA Removal Kit (#AM1906) from ThermoFisher. The protocol is based on the one provided by the kit, described in the appendix (App. 5).

The retrotranscription was performed with the RevertAid RT Reverse Transcription Kit (#K1691) and following the protocol provided (App. 6).

An amplification by the GoTaq® G2 DNA Polymerase (#M7841) followed by a migration on agarose gel at 1 % also allowed to confirm the good realization of the DNase treatment and the retrotranscription (App. 7).

2.2.3.3. quantitative Polymerase Chain Reaction

A qPCR analysis was performed on the basis of several genes of interest (GOI). EF1a is used as a housekeeping gene (HKG) to standardize the expression of the GOI. Primers were selected through NCBI and tested for their efficiency for these genes. Samples from all 6 conditions (3 time points and 2 permethrin concentrations) were tested (N = 8 per condition) and each sample was tested in triplicate.

The genes of interest analyzed were: GAPDH, IGF-2, keap1b, mTOR, SIRT1, SIRT2, PSMA1, PSMB1, NEF2. These are known to be involved in the control of cell growth and oxidative stress (Arif *et al*., 2022; Husak & Lailvaux, 2022; Nguyen *et al*., 2020; Singh & Ubaid, 2020; Udroiu *et al*., 2022; Yang *et al*., 2022). However, some results showed a lack of efficiency or specificity during qPCR, so the affected genes were removed. The genes eventually reported are the following: GAPDH, IGF-2, mTOR, SIRT1, SIRT2, PSMB1.

Real-time PCR tests and plate filling were performed with the SsoAdvanced Universal SYBR® Green Supermix (#1725274) from Bio-Rad Laboratories according to the attached protocol (App. 8). Thermal cycler was used and the results were analyzed based on the Pfafll method.

2.3. Statistical analysis

Statistical analyses were performed on R software (version 4.1.1). Data were studied by creating and comparing nested models: linear model (LM) for length measurement, generalized linear model (GLM) with Poisson distribution for egg count, lm for expression of GOI. The conditions for applying parametric tests, i.e., homogeneity of variances and normality of residuals (for LM) or overdispersion (for GLM), were checked and the data were transformed if these conditions were not met. The ideal model was then subjected to an Anova II test

(package *car*) to identify the explanatory variables with a significant impact on the response variable.

Survival in the different groups was analyzed using a Log-Rank test and the "survdiff" function (*survival* package). The lifespan of each individual was taken into account to make a survival curve. A GLM with binomial distribution was also used to study the mortality after 146 days.

The reproductive indexes were analyzed based on their mean following permethrin exposure. Normality and homoscedacity were tested to determine the type of test used.

C. Results

1. Effect of reproduction on aging and life history traits

1.1. Survival analysis

Survival analysis by the Kaplan Meier curve shows significant differences in survival for the 4 studied groups (Log-Rank test; Chi-squared = 13.47; Df = 3; 0.01 > p > 0.001; Fig. 3). Until day 60, little mortality was observed in the 4 groups. After that, the curves of the community individuals (both 0 µg/L and 100 µg/L permethrin) decreased significantly faster than the curves of the isolated individuals (both 0 µg/L and 100 µg/L permethrin) (Log-Rank test; Chi-squared = 12.94; Df = 1; p < 0.001). Therefore, individuals in the community had on average a shorter lifespan than the isolated individuals. In the community group, 50 % of the individuals died after only 95 days, whereas in the group of isolated individuals, the probability of survival dropped below 50 % after about 142 days. However, no effect of permethrin exposure concentration was shown on survival (Log-Rank test; Chi-squared = 0.08; Df = 1; p = 0.77).

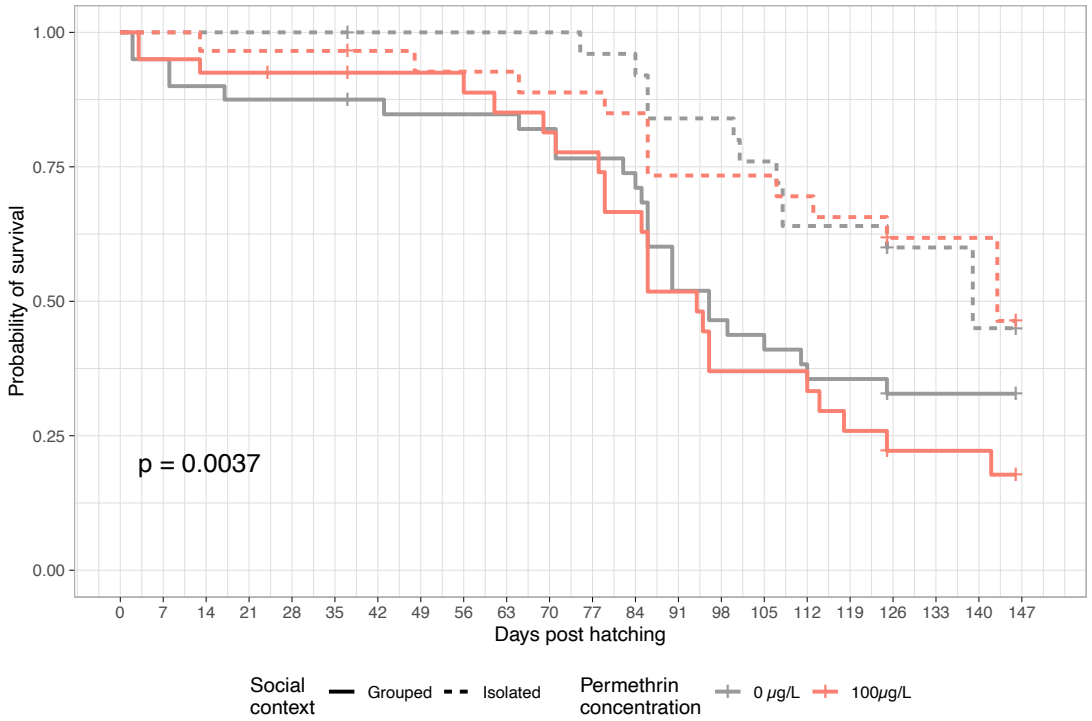

Figure 3: Kaplan Meier survival curve of the 4 groups tested i) community group exposed to 0 µg/L of permethrin (control; N = 40), ii) community groups exposed to 100 µg/L of permethrin (N = 40), iii) control isolated individuals (N = 27), and iv) isolated fish exposed to 100 µg/L of permethrin (N = 29). The p-value is the Log-Rank test performed on the difference of the 4 curves.

After 146 days (about 21 weeks of life), the probability of survival was significantly different between the isolated and the grouped individuals (GLM with binomial distribution; Chi-squared = 17.09; Df = 1; $p < 0.001$). Actually, isolated individuals have a higher probability of survival (0.46) than grouped individuals (0.24). Other effects tested were permethrin concentration (GLM with binomial distribution; Chi-squared = 1.61; Df = 1; p = 0.20), cohort effect (GLM with binomial distribution; Chi-squared = 1.82; Df = 1; p = 0. 18) and the interaction between social context and permethrin concentration (GLM with binomial distribution; Chi-squared = 2.91; Df = 1; p = 0.09) but none of these effects had a significant impact on survival at the end of the experiment.

1.2. Morphological analysis: length

The total size of individuals was measured at five time-points and analyzed on the basis of different effects: social context, permethrin concentration, cohort and the interaction social context and permethrin concentration (Tab. 1; Fig. 4).

Table 1: Summary of the studied effects on the size of individuals at different time-points.

	0 wph	**1 wph**	**5 wph**	**12 wph**	**17 wph**
Social context	LM F value = 1.88 Df = 1 p = 0.17	LM F value = 49.05 Df = 1 **p < 0.001**	LM F value = 21.77 Df = 1 **p < 0.001**	LM F value = 1.64 Df = 1 p = 0.21	LM F value = 23.11 Df = 1 **p < 0.001**
Permethrin concentration	LM F value = 1.23 Df = 1 p = 0.27	LM F value = 1.88 Df = 1 p = 0.17	LM F value = 5.30 Df = 1 **0.01 < p < 0.05**	LM F value = 0.58 Df = 1 p = 0.45	LM F value = 18.59 Df = 1 **0.001 < p < 0.01**
Cohort	LM F value = 17.53 Df = 1 **p < 0.001**	LM F value = 97.01 Df = 1 **p < 0.001**	LM F value = 523.90 Df = 1 **p < 0.001**	LM F value = 3.20 Df = 1 **0.001 < p < 0.01**	LM F value = 28.87 Df = 1 **p < 0.001**
Social context and permethrin concentration interaction	LM F value = 0.0024 Df = 1 p = 0.96	LM F value = 0.32 Df = 1 p = 0.57	LM F value = 5.08 Df = 1 **0.01 < p < 0.05**	LM F value = 0.037 Df = 1 p = 0.85	LM F value = 20.98 Df = 1 **0.001 < p < 0.01**

At haching (0 wph), size differed only between the two cohorts (LM; F value = 17.62; Df = 1; $p < 0.001$). Individuals in the second cohort remained smaller on average compared to those in the first. From the 1st wph (LM; F value at 1 wph = 97.50; Df = 1; $p < 0.001$), i.e., at the end of exposure, to the 5th wph (LM; F value at 5 wph = 523.90; Df = 1; p < 0. 001), the

cohort effect is still very present but the social context also strongly impacts the length of individuals at the 1st wph (LM; F value = 39.70; Df = 1; $p < 0.001$) and the 5th wph (LM; F value = 21.77; Df = 1; $p < 0.001$). At 1 and 5 wph, grouped individuals were significantly smaller in the isolated condition (9.27 mm and 22.65 mm respectively) than in the grouped condition (10.08 mm and 23.27 mm respectively). A concentration effect is added at 5 wph (LM; F value = 5.30; Df = 1; $0.01 < p < 0.05$). The presence of permethrin leads to a decrease in size (22.39 mm) compared to control individuals (23.84 mm). This also leads to an interaction effect between context and concentration (LM; F value = 5.08; Df = 1; $0.01 < p < 0.05$).

At 12 wph, all effects were reduced and length varies only weakly with cohort (LM; F value = 3.20; Df = 1; $0.001 < p < 0.01$). The other effects showed no significant impact.

At 17 wph, length was affected by all effects tested. Social context (LM; F value = 23.11; Df = 1; $p < 0.001$) and cohort (LM; F value = 28.87; Df = 1; $p < 0.001$) strongly affect this morphological data. In addition, permethrin concentration (LM; F value = 18.59; Df = 1; $0.001 < p < 0.01$) and social context/permethrin concentration interaction (LM; F value = 20.98; Df = 1; $0.001 < p < 0.01$) also have a fairly significant effect.

It is also important to note that at weeks 0 and 1, all individuals were measured because sexual differentiation had not yet occurred, whereas for the rest of the analysis, only males were tested.

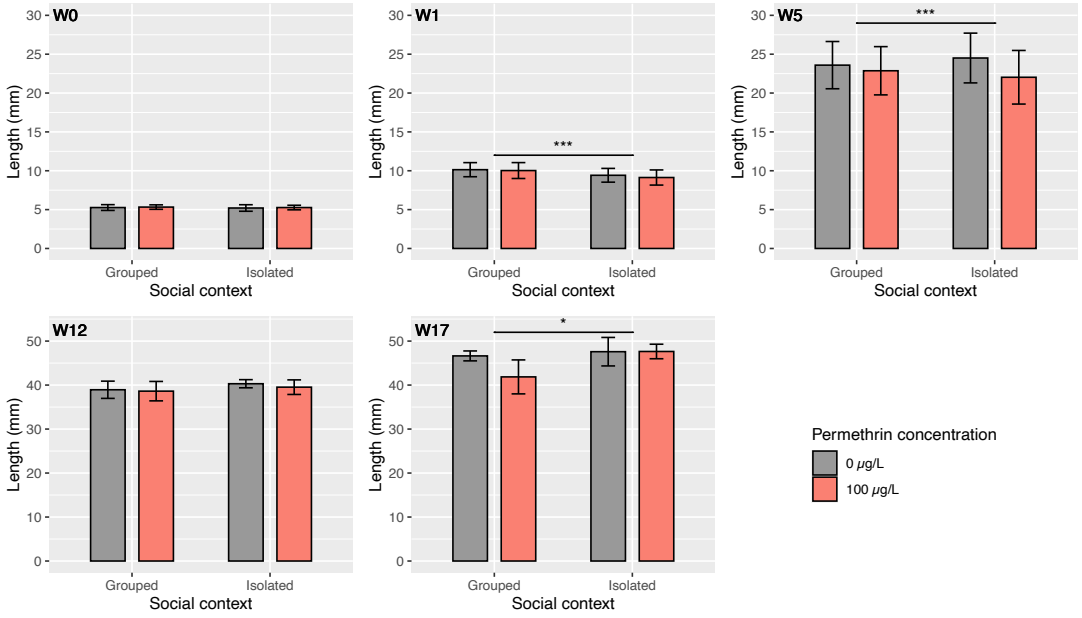

*Figure 4: Length according to the social condition and the exposure concentration. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. The means and standard deviations of the sizes according to these two conditions are shown in Table 2.*

Table 2: Summary of means and standard deviations of sizes measured in millimeters according to social context and permethrin concentration at each time point.

Social context	Permethrin concentration	Length at 0 wph (mm)	Length at 1 wph (mm)	Length at 5 wph (mm)	Length at 12 wph (mm)	Length at 17 wph (mm)
Grouped	0 μg/L	Mean = 5.26 Sd = 0.37	Mean = 10.13 Sd = 0.90	Mean = 23.59 Sd = 3.03	Mean = 38.93 Sd = 1.95	Mean = 46.63 Sd = 1.12
Grouped	100 μg/L	Mean = 5.32 Sd = 0.28	Mean = 10.02 Sd = 1.02	Mean = 22.86 Sd = 3.10	Mean = 38.61 Sd = 2.20	Mean = 41.86 Sd = 3.86
Isolated	0 μg/L	Mean = 5.20 Sd = 0.42	Mean = 9.41 Sd = 0.88	Mean = 24.50 Sd = 3.20	Mean = 40.30 Sd = 0.92	Mean = 47.59 Sd = 3.23
Isolated	100 μg/L	Mean = 5.26 Sd = 0.29	Mean = 9.13 Sd = 0.97	Mean = 22.03 Sd = 3.44	Mean = 39.52 Sd = 1.65	Mean = 47.63 Sd = 1.65

2. Environmental effects on reproduction

2.1. Daily eggs collection

The total number of eggs collected from week 7 to week 18 varied with the age of the individuals (Anova II; Chi-squared = 79.01; Df = 1; $p < 0.001$). Around the 7th wph, *N. furzeri* began to lay eggs and the number of eggs laid increased in both conditions until the 10th wph. The curves then separated and individuals exposed to permethrin laid an average of 10 eggs per day. An effect of exposure concentration of individuals was demonstrated (GLM with Poisson distribution; Chi-squared = 10.03; Df = 1; $0.01 > p > 0.001$; Fig. 5A). In the control group, the number of eggs continues to increase until reaching an average of about 25 eggs around 15 wph before decreasing. Individuals exposed to permethrin laid significantly fewer eggs than the control group. No interaction effect between age and permethrin was demonstrated in this study (GLM with Poisson distribution; Chi-squared = 1.631; Df = 1; $p = 0.20$).

The cumulative number of eggs per week was also measured for each permethrin concentration. The average number of eggs laid in total by a pair at the end of 18 weeks was 77.91 eggs for the permethrin-exposed individuals while the control individuals laid double that number, 158.38 eggs (Fig. 5B).

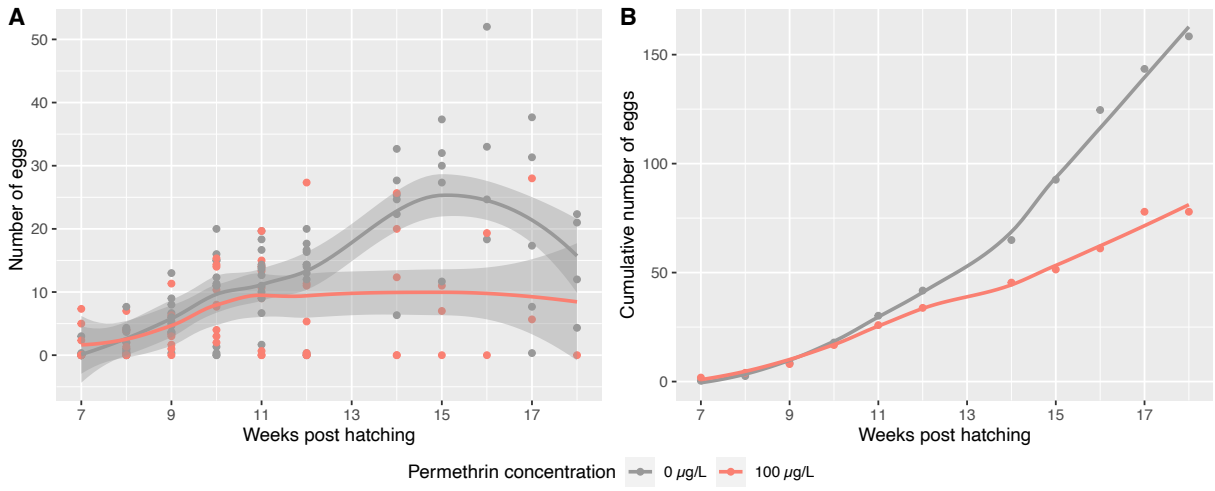

Figure 5: A: Total number of eggs harvested per couple per day according to the age of the parents. B: Cumulative number of eggs collected per couple per day according to the age of the individuals. The number of pairs tested varies during the experiment due to mortality. At 7 wph, N = 9 and 8 for the group exposed to 0 µg/L and 100 µg/L permethrin, respectively. At 18 wph, N is only 4 and 1 for the 0 µg/L and 100 µg/L permethrin group, respectively.

2.2. Reproductive indexes

Only the number of eggs laid and the fertility index was significantly different between exposure concentrations (Kruskal-Walli's test; Chi-squared = 12.12; Df = 1; $p < 0.001$). Individuals exposed to permethrin laid fewer eggs in total (6.93) than control individuals (11.39).

Fertility (Kruskal-Walli's test; Chi-squared = 2.85; Df = 1; p = 0.091), fertilization (Kruskal-Walli's test; Chi-squared = 0.29; Df = 1; p = 0.29) and developmental (Kruskal-Walli's test; Chi-squared = 0. 84; Df = 1; p = 0.84) indexes when exposed to permethrin were not significantly different from control measurements (Fig. 6). The means are similar between concentrations. The averages of controls and permethrin-exposed individuals are respectively 0.89 and 0.90 for the fertility index and 0.86 and 0.85 for fertilization index. The development index only reaches about 0.42 and 0.35 for the two conditions.

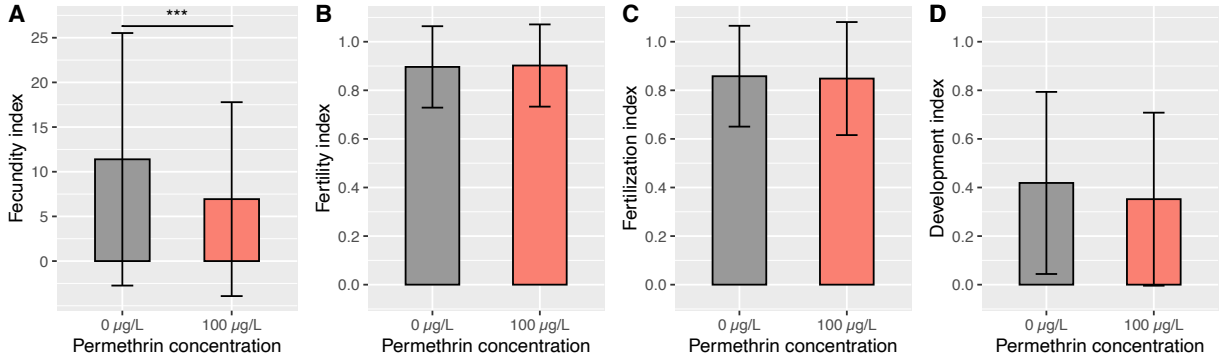

Figure 6: Reproduction indexes for the two exposure concentrations. A: Fecundity index. B: Fertility index. C: Fertilization index. D: Development index.

2.3. Expression of genes of interest (GOI)

The expression of GOI was studied as a function of permethrin concentration, parental age and the interaction of these two factors. The qPCR results were analyzed by the Pfafll method according to the efficiency of the primers used (App. 12).

The genes of interest were GAPDH, IGF-2, mTOR, SIRT1, SIRT2, PSMB1. This analysis showed no change in the expression of the six GOI (N = 8; Fig. 7). Based on the mean and standard deviation values (Tab. 3), it can be observed that the expression variability is very high at the level of several genes of interest which could explain the lack of clear trends.

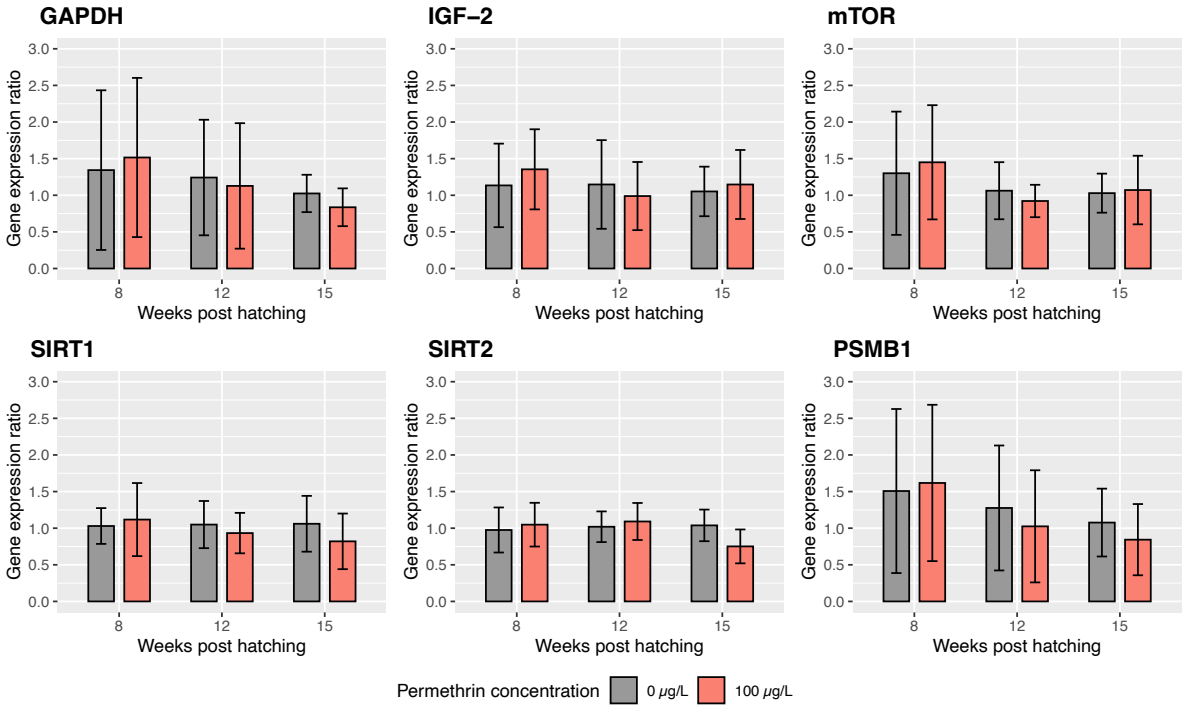

Figure 7: Gene expression ratio of the genes of interest. The qPCR analysis showed no difference between the expression of the genes. The summary table of means and standard deviations can be found in the Appendix (App. 13).

Table 3: Summary of the effects studied on the expression ratio of the genes of interest.

	GAPDH	IGF-2	mTOR	SIRT1	SIRT2	PSMB1
Permethrin concentration	LM F value = 0.19 Df = 1 p = 0.66	LM F value = 0.053 Df = 1 p = 0.82	LM F value = 0.028 Df = 1 p = 0.87	LM F value = 0.91 Df = 1 p = 0.34	LM F value = 0.59 Df = 1 p = 0.45	LM F value = 0.43 Df = 1 p = 0.51
Parental age	LM F value = 0.57 Df = 2 p = 0.57	LM F value = 0.52 Df = 2 p = 0.60	LM F value = 1.19 Df = 2 p = 0.31	LM F value = 0.61 Df = 2 p = 0.59	LM F value = 1.42 Df = 2 p = 0.25	LM F value = 1.66 Df = 2 p = 0.20
Age and permethrin concentration interaction	LM F value = 0.64 Df = 2 p = 0.53	LM F value = 0.39 Df = 2 p = 0.67	LM F value = 0.22 Df = 2 p = 0.80	LM F value = 1.00 Df = 2 p = 0.37	LM F value = 2.37 Df = 2 p = 0.11	LM F value = 0.64 Df = 2 p = 0.53

D. Discussion

This study aimed to characterize how social context and early exposure to neurotoxic compound could interact to influence aging and life history traits in fish. The turquoise killifish *Nothobranchius furzeri* was used as a model to perform a longitudinal analysis at the individual level. This emerging model allowed the concept of senescence to be incorporated into this study.

The first objective was to identify potential links between aging and social context. Survival analysis showed that communal living resulted in a shorter lifespan. After 21 weeks of life, the probability of survival is much higher in the isolated individuals. A negative correlation is well present between communal living and longevity in *N. furzeri*, mortality seems to be accelerated with this lifestyle. Reproduction is one of the important functions present during communal living. Other studies have shown similar results in terms of the impact of reproduction on survival and size. The negative correlation between survival and reproduction has already been demonstrated in a wide variety of model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Musca domestica* and *Callosobruchus maculatus* (Graf *et al*., 2010; Pontzer & McGrosky, 2022). This would be due to a trade-off between current reproductive investment and future reproduction. Early sexual maturity reduces the lifespan of individuals. Therefore, they will have less time to reproduce in the future and their chances of future reproduction will decrease (Soumaila *et al*., 2022).

In addition to lifespan, growth was another life history trait impacted by communal living. The link between mortality and size remains complicated to elucidate but the impact of social contacts on size seems unequivocal. In fact, this lifestyle caused a slowing of growth early in the lives of individuals that appeared to be caught up from the 12th wph. The results of 17th wph still showed a slight effect of social context, but following the death of many individuals, the number measured in the community group decreased sharply in the 100 μg/L permethrin condition (N = 2). It is therefore best to remain critical of these data. According to similar studies, reproductive effort interacts with somatic growth and leads to a decrease in body size (Graf *et al*., 2010; Thoré *et al*.., 2020). In our study, a small effect was observed in males early in life, but Graf *et al*. (2010) demonstrated that this trade-off would be related to reproduction, a function allowed during communal living but absent in isolated individuals. Females would therefore be more impacted as they provide a greater energy investment for germ cell production (Graf *et al*., 2010; Mason *et al*., 2022).

Graf *et al*. (2010) also attempted to show a difference in survival upon sex separation. However, this experiment showed no difference in the lifespan of the different groups (Graf *et al*., 2010). As discussed earlier, reproduction is part of the processes involved in living in a community. Graf *et al*. (2010) study the direct impact of this reproduction by dissociating it from the overall community life style. Compared to this study, the major difference in our analysis comes from the fact that the fish intended not to reproduce were isolated from social contact. This could explain the differences in the results obtained, because beyond reproduction, living in a community leads to the appearance of multiple energy-consuming behaviors that are at the origin of social stress. Group living leads to sexual harassment of males towards females and reduces foraging efficiency (Graf *et al*., 2010; Thoré *et al*., 2020). Graf *et al*. (2010) also discuss the importance of nutrition in the survival-reproduction trade-off. In our case, the fish were fed *ad libitum*, so nutrition did not appear to be a limiting factor, but this new element could be responsible for a 50 % decrease in foraging. Males are also affected by the costs of sexual harassment, conflict for females, and exploration of the habitat to find a mate (Thoré *et al*., 2020). Intra-sexual social stress is also important in males even in the absence of females. In order to define hierarchy, males repeatedly physically attack each other, which is very energetically costly (Graf *et al*., 2010). All these expenses do not occur when individuals are totally isolated as in our experiment.

The main hypothesis explaining the impact of social context would be an energy trade-off between reproduction and life history traits such as survival and growth (Pontzer & McGrosky, 2022). The energetic allocation for reproduction would actually be diverted from maintenance which would lead to faster senescence (Pontzer & McGrosky, 2022). The principle would be the same for somatic growth and body size (Thoré *et al*., 2020). Wu *et al*. (2022) have already identified a gene that would be involved in the reproduction-longevity trade-off in *Caenorhabditis elegans*. The trl-1 gene would be responsible of the allocation of energy between these two processes. Its mutant therefore expresses an increase in reproduction at the expense of longevity (Wu *et al*., 2022). To our knowledge, no study has investigated the presence and the role of this gene in *N. furzeri*. This could help confirm the existence of a trade-off between these processes in this species and open up the question of its involvement in the rapid senescence characteristic of *N. furzeri*. A molecular analysis would be crucial to support this theory based on energy allocation. This research could also provide insight into the control of inter-individual or inter-lineage variability.

The study of reproduction in *N. furzeri* showed that this process was also impacted by senescence. Fecundity data collected showed variation in the number of eggs laid over the lifetime of *N. furzeri*. These fish rapidly reach sexual maturity and begin to lay eggs around their 7th week of life. After this decisive event in the reproductive life, the number of offspring will continue to vary over the life. Around the 15th wph egg production reached its maximum and will then decrease until reproduction stops and death occurs. Age had a direct effect on the reproductive fitness of *N. furzeri* and reproductive senescence played an important role in this process. This concept reveals a trade-off between current and future reproduction. Fecundation is then impacted by the age of the individuals (Žák & Reichard, 2021). Although *N. furzeri* has a shorter lifespan, all age-related phenotypes, such as sensory and fertility decline, are still observed (Api *et al.*, 2018). Its life cycle including reproductive abilities are actually accelerated. Žák & Reichard (2021) have shown that reproduction does not occur in the same way throughout life but also that reproductive senescence varies greatly between individuals. In our study, inter-individual variability was observed in the measure of fecundity. The number of eggs laid varied strongly between pairs within a week of life. In the generation of offspring, as said before, females are responsible for the production of germ cells, so the number of eggs will be more impacted by maternal age (Api *et al.*, 2018). A high early fecundity would then have effects on late life fecundity and survival. It has often been reported that fish and organisms with post-mature growth escape the aging process and are not subject to reproductive senescence (Žák & Reichard, 2021). This longitudinal study demonstrated the existence of reproductive senescence in fishes, even in the short-lived turquoise killifish.

Potential parental effects on the next generation were tested by quantifying the expression of several genes that are involved in aging function (GAPDH, IGF-2, mTOR, SIRT1, SIRT2, PSMB1). They are responsible for cell growth and cycle, DNA repair, intracellular protein degradation, and apoptosis. The results showed that the age of the parents did not impact the expression of these genes. However, it is known that parental age can impact the biochemical composition at the level of membrane proteins (lipids, proteins and phosphate groups) and the amount of stored energy (Api *et al.*, 2018). These parameters are critical in understanding reproduction. Aged parents can therefore cause a slowdown of the development and the growth rate of their offspring via macromolecules stored within the egg (Api *et al.*, 2018).

In addition to social context and reproduction, life-history traits and aging may also be sensitive to environmental stressors. Our second objective was to highlight the effect of permethrin, a neurotoxic compound, on *N. furzeri* individuals throughout their life. A randomly selected group was exposed to permethrin, a neurotoxicant chosen because of its widespread presence in the pesticide market.

First, exposure to permethrin influenced reproduction: the presence of permethrin caused a decrease in the quantity of laid eggs. The control individuals laid on average twice as many eggs as the individuals exposed to permethrin. However, analysis of eggs quality indexes showed that the quality aspect of the eggs was not affected. Overall, our data suggest a decrease in the number of eggs that may reached the end of their development and were ready to hatch. Several studies have supported the harmful nature of permethrin for reproduction. In zebrafish, it impacts fertility and endocrine signaling (Blanc *et al*., 2020). Sex hormones are altered with antiandrogenic activity in males and estrogenic activity in females (Blanc *et al*., 2020; Hénault-Ethier & Gagné, 2016; Tange *et al*., 2014). This results in a decrease of testosterone concentration and leads to a decrease in sperm production in males (Hénault-Ethier & Gagné, 2016). Permethrin therefore has a direct effect on reproduction and gamete production. According to Sokolova (2021), this could also be explained by a disturbance in energy allocation. Exposure to toxic substances represents a chemical stress that is perceived by the organism and affects cellular homeostasis. The body must therefore set up energy-intensive elimination and detoxification mechanisms. The necessary energy would in fact be diverted from other processes which would affect their functioning (Sokolova, 2021).

Finally, the size of the individuals, their survival and the impact of permethrin on the next generation were also studied. Only a slight effect on early life size was found to be significant. Other traits did not seem to be affected by the presence of permethrin in the larval environment. However, Blanc *et al*. (2020) observed a transgenerational decrease in larval activity in the presence of permethrin. Permethrin would be able to induce epigenetic perturbations transmissible to subsequent generations and modify the phenotype of the offspring (Harney *et al*., 2022). Low doses of pollutants have been shown to cause differences in cytosine methylation in *Daphnia pulex*. This would lead to long-term persistent effects in exposed individuals (Harney *et al*., 2022). It would therefore be interesting to take this analysis further and characterize the transgenerational impact beyond embryonic development at the epigenetic level.

E. Conclusion

By investigating the role of social context on life history traits and aging in *N. furzeri*, we demonstrated that lifestyle is able to influence the lifespan of individuals. In fact, communal living significantly reduces longevity in this species. Our data also highlighted the existence of a trade-off between community living and longevity in this fish. A negative correlation would exist between the behaviors and functions included in this lifestyle and the lifespan. Future studies would benefit from examining the involvement of energy allocation in these processes. Identification and analysis of the genes responsible for these trade-offs would be essential to identify the underlying mechanisms and confirm this theory. The key principle of energy allocation is based on the combined analysis of all biological processes. If any one of them is ignored, the resulting evolutionary conclusions could be misleading (Husak & Lailvaux, 2022). In this short-lived fish, known for its rapid senescence, it would also be interesting to find out if this difference in longevity is related to a more accelerated senescence. The use of a senescence biomarker such as lipofuscin would be a possible technique (López-Otín *et al.*, 2023).

Moreover, reproduction is an integral part of group life and is also impacted by environmental parameters. Permethrin is a toxic molecule that altered reproduction and more precisely the fecundity of *N. furzeri*. The number of offspring is divided by two when individuals have been early exposed to permethrin. Moreover, the main effects observed following this exposure are delayed effects, mainly on reproduction. It would be particularly interesting to investigate the nature of the mechanisms underlying these delayed effects. Epigenetics is an avenue to be explored in this sense in order to highlight potential modifications in the DNA structure caused by permethrin.

The results obtained open additional questions on the effects of aging and social conditions on reproductive strategy, lifespan and transgenerational effects. *N. furzeri* is a key model in long-term research, with its very rapid life cycle, it makes analysis of lifespan and delayed effects of early exposure all the more accessible (Cellerino *et al.*, 2016; Žák & Reichard, 2021). This emerging model needs to take hold in senescence research. In this study, it was highlighted that individual variation is very present despite the general trend observed. The use of a harmless identification method would bring an individual dimension to this longitudinal analysis and the ability to correlate a particular phenotype with its genotype.

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G. Annexes

1. Additional information: state of the art

Environmental variations and context

The theory of the evolution was discovered in the 18th century. This new theory was the first to write about the importance of the environment on species (Henet, 2020). The genetic material is complex and probably responsible for the majority of the characteristics that an individual has. The genetic diversity of species allows natural selection to interact with this material acquired from embryogenesis. The result of this selective pressure is the evolution of species. Some individuals will then acquire increasingly more traits in line with their environment. Evolution thus shows that the environment plays an essential role in the distribution of adapted traits among species (Henet, 2020).

The fitness is one of the main forces that interacts with natural selection. This process is also called the reproductive success and refers to the capacity of a genotype to reproduce. The primary goal of an organism is to spread its genes throughout the population. The reproduction is thus very important to reach this aim. Life history traits also play an essential role, they are defined by Lenoir (2004) as "the set of major events during the life of an individual that contribute directly to the production and survival of offspring" (Lenoir, 2004). Development, growth, reproduction and survival are part of life traits. The success of each individual's life strategy is therefore dependent on the trade-off that is created between these different actions (Api *et al*., 2018). Reproductive success is for this reason directly impacted by reproductive traits but also by the energetic allocation that will be given to this trait (Api *et al*., 2018; Henet, 2020). This allocation varies with the age of the individual as well. Young females will allocate more energy to their reproduction than old females who have certainly already reproduced (Api *et al*., 2018). So, life history trait theory allows to associate the evolutionary aspect with ecological and physiological components (Graf, Cellerino and Englert 2010).

A temporary change in environmental conditions can impact the life history traits of the population present at that location. The effects can also be observed on the offspring of this generation (Hellou, 2011).

Under hostile environmental conditions, the age of sexual maturation may also be altered. In this case, selective pressure on reproductive traits is important because the risk of dying is higher in this environment (Api *et al*., 2018). Parents will produce offspring more quickly to maximize their reproductive success despite changing conditions. The next offspring will then have an earlier sexual maturity in order to continue in this direction and ensure their reproduction (Hellou, 2011; Terzibasi *et al*., 2008).

When an individual evolves in an environment, this one will obviously interact with the organism. The presence of food is one of the conditions that can vary in the environment, it is not everywhere distributed in the same quantity and quality. However, the available resources can modulate the importance that will be given to reproduction by the energy used for it. This immediately impacts the reproductive fitness and survival chances of an embryo before it even begins to develop. This process is called parental influence and is based on the storage of resources by the female in their egg. Other factors such as age or environmental conditions can also influence energy storage in eggs (Api *et al*., 2018). In fact, these examples show that the environment has a significant influence on the life history traits of individuals living in it.

Energy supply is therefore the main limitation in life history traits. Moreover, reproduction appears to represent a significant and essential cost in the life of organisms. Trade-offs exist in the allocation of this energy. The impact of reproductive costs on lifespan and thus aging is one of the most studied (Graf, Cellerino and Englert 2010). Aging is the process that leads to the gradual decline of the physiological function and the increasing vulnerability of the organism until death (Henet 2020; Liu and Sabatini 2020). Several theories attempt to explain this phenomenon that all species experience. Defenders of the classical evolutionary theory of aging believe that a decrease in the strength of selection at older ages leads to senescence. Senescence is no longer as sensitive to selection as other traits present earlier in the life of individuals (Terzibasi *et al*., 2008). As explained above, the decrease in selective strength comes from the fact that almost every offspring will be produced before senescence. So, the selection that takes place at the end of the individual's life will no longer have a way to affect its offspring. The strength of selection is therefore weaker when reproduction has already occurred (Henet, 2020; Terzibasi *et al*., 2008). This hypothesis meets criticism based on the example of Drosophila. When this insect sees its mortality increase in younger individuals, maturation will evolve to also arrive more quickly. Senescence follows the same path, showing that it is always the target of selection (Terzibasi *et al*., 2008). The turnover of older individuals

by younger ones or the influence of predation on delayed senescence are other theories that have been put forward. Aging may also be the result of an energy trade-off between reproduction and cell maintenance. Energy use in reproduction would therefore cause a decline in maintenance (Henet, 2020; Terzibasi *et al*., 2008). These theories are based on different principles, but this does not mean that they are mutually exclusive.

Anthropogenic condition: the presence of permethrin

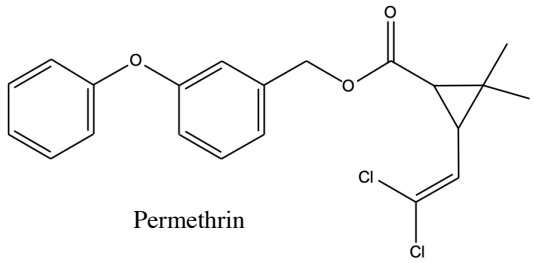
Pesticides have been around for a long time, many substances are used since the last XIX century (Fourdinier, 2017). The pyrethrin was discovered in 1920 and was at the origin of a new family of synthetic pesticides, the pyrethroids. The natural origin of this molecule is the pyrethrum, which is produced by plants in the chrysanthemum family (Aznar-Alemany and Eljarrat 2020; Goulin 2016). In 1949, the first type I pyrethroid was created, it was allethrin. Then, this family of insecticides continued to grow. A few years later, in 1970, the molecule of permethrin is also created. At first, it was used as a simple household product, but its antiparasitic properties allowed it to become indispensable in agriculture (Aznar-Alemany and Eljarrat 2020; Goulin 2016).

At that time, organophosphorus products were the mainly-used pesticides, but the effective dose for crop protection was often close to the lethal dose so that, their use remained rather risky (Goulin, 2016). Pyrethroids have therefore taken over in agriculture by gradually replacing organophosphates in this field. They have many advantages such as a better photostability, more selective toxicity as well as great efficacy. This last characteristic have helped pyrethroids to currently represent a quarter of the pesticides used (Aznar-Alemany and Eljarrat 2020; Goulin 2016).

Pyrethroids are weighty molecules, their weight reaches 300 g/mol. Their logarithm octanol-water partition coefficient (K_{ow}) can range from 4 to 7, making them very hydrophobic. They are also quite photosensitive and can be metabolized in the liver in mammals. These characteristics make the degradation time (TD_{50}) less than 60 days. Therefore, these pesticides are not known to persist in the environment or to bioaccumulate (Aznar-Alemany and Eljarrat 2020). Permethrin is also very sensitive to ultraviolet light, which rapidly degrades it. So, its half-life in the soil is less than 28 days and it can decrease further in the presence of microorganisms or depending on the environmental conditions. When the permethrin is watered down, it persists only a few days. However, this also explains that it will be degraded less quickly if this molecule is sheltered from light as in the domestic environment (Goulin, 2016).

Pyrethroids are used in many areas as commercial products, household products and even in veterinary medicine as an external antiparasitic to control scabies and lice. But in 1962, Rachel Carson warned of the toxicity of excessively-used pesticides. She published her book "Silent Spring" which denounced these practices. It was then and it is still essential to conduct toxicological studies when a new pesticide is put on the market (Aznar-Alemany and Eljarrat 2020). That is why several toxic effects are now associated with pyrethroids, such as neurotoxicity, carcinogenicity, immunosuppression and damage to the reproductive system (Aznar-Alemany and Eljarrat 2020).

As mentioned above, permethrin belongs to this family, it is a type I pyrethroid (Fig. 8). This pesticide has a wide spectrum of action in insects and a fairly small amount can already generate significant effects (Aznar-Alemany and Eljarrat 2020; Goulin 2016). It is a neurotoxic substance that has different characteristics from toxic substances using other modes of action. They are found, in general, in fairly low concentrations in the environment but their impacts can even so be significant (Thoré *et al*., 2020).



Permethrin

Figure 8: Schematic representation of the permethrin. This synthetic molecule comprises an acid group and an alcohol group linked by an ester bond. This configuration is characteristic of type I pyrethroid molecules (According to Aznar-Alemany & Eljarrat, 2020; Goulin, 2016).

Permethrin acts on the central and peripheral nervous system. It attacks the activity of voltage-gated sodium or calcium channels and calcium-ATP[1]ase pumps (Goulin, 2016). The role of these channels is to transport ions across the cell membrane. By disrupting this activity, permethrin reduces the threshold of the potential of action near them and causes repeated stimulation at the level of nerve and muscle cells (Aznar-Alemany and Eljarrat 2020). This membrane depolarization leads to other consequences such as altered neurotransmitter release

[1] Adenosine TriPhopshate.

or GABA[2]-ergic transmission (Goulin, 2016). This neural stimulation pathway has been highly conserved by natural selection, so it is present in many species. Therefore, the presence of this substance in the natural environment reflects a high risk for non-targeted species. They can also suffer the same process. Permethrin can interact with insect-like receptors and thus induce the same consequences in organisms that are not part of the targeted species (Thoré *et al*., 2020). In addition to neurotoxicity, other effects of this molecule have also been observed. Hepatic, cardiac or immune toxicity cannot be excluded (Goulin, 2016). Estrogenic and anti-androgenic actions also impact the reproductive and endocrine system (Ravula and Yenugu 2019; Tange *et al.* 2014).

Insects, the targeted organisms, still remain 2250 times more sensitive to this substance than mammals, this is related to their physiognomy. They have more sensitive sodium channels, are smaller and have a lower body temperature as well. An important point is also that mammals have a fairly low skin absorption. In the latter, it is oral absorption or inhalation that is the most important input channel. But that is the opposite with insects (Aznar-Alemany and Eljarrat 2020; Goulin 2016). Still, these other two absorption routes should not be underestimated because pyrethroid residues can be found in treated products (Aznar-Alemany and Eljarrat 2020).

In aquatic organisms, the presence of permethrin causes epigenetic deregulation and a decrease in the number of eggs laid by the female. This is a result of its endocrine disrupting properties (Blanc *et al.* 2020). The study by Blanc *et al*. (2020) also demonstrated a transgenerational effect on zebrafish reproduction (Fig. 9).

[2] Gamma-AminoButyric Acid.

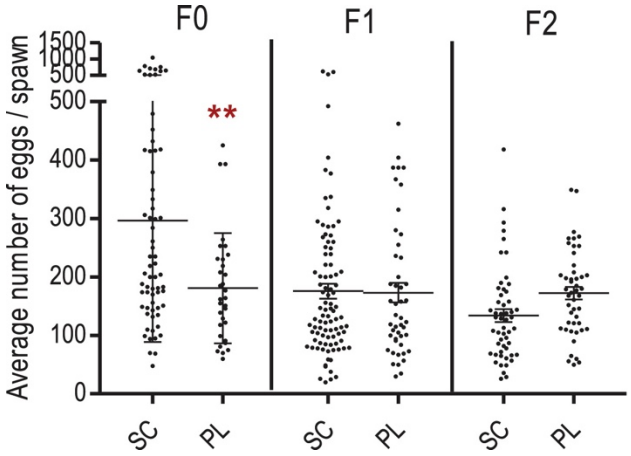


*Figure 9: Number of eggs laid per female for the different generations. **: p < 0,01. PL: Permethrin 1 µg/L; SC: Solvent control (DMSO 0.01%). (Blanc et al., 2020).*

In the figure above, the number of eggs that are laid is counted for females of different generations. The F0 generation was exposed to the neurotoxicant during the first 28 days of its life. There is a significant difference in the studied parameter in F0 females, exposed females produce less eggs. The F1 and F2 females maintained an egg production that is lower than normal (F0 control) while they were not exposed to permethrin at any time (Blanc *et al*., 2020). The nervous system is very sensitive to environmental conditions such as the presence of permethrin. This sensitivity is even greater during embryonic development. So, early exposure may result in delayed behavioral effects. Alteration of neuronal function can be continuous and induce persistent neurodegenerative diseases (Blanc *et al*., 2020; Nunes *et al*., 2019). The neurobehavioral phenotype is disrupted and an increase in anxiety-like behavior in zebrafish larvae has already been observed at sublethal concentrations of 25 and 50 µg/L (Blanc *et al*., 2020; Nunes *et al*., 2019).

Other identified effects are a production of ROS (Reactive Oxygen Species), increased oxidative activity and fragmentation. These impacts can lead to cell death (Nunes *et al*., 2019). A 24-hour survival curve determined that the LC50 is 108 µg/L in zebrafish larvae. This measure gives the concentration of toxic needed to cause the death of the 50% of the tested individuals. The first effects are observed above the No Observed Effect Concentration (NOEC). In the view of Nunes et al. (2019), the NOEC of the permethrin is 50 µg/L (Nunes *et al*., 2019). Neurotoxic substances are known to primarily affect behavior (Thoré *et al*., 2020). Subsequently, a behavioral change may alter the population's rhythm of life and impact its reproduction or balance with the environment. Another poorly studied parameter is the chronic exposure of organisms. Nevertheless, this is what happens most of the time, pesticides remain

in the environment for a long time. The presence of other toxic substances can also cause so-called "cocktail" effects that are even more harmful to organisms. However, chronic effects as well as interactions between toxicants remain rarely studied in ecotoxicology (Ravula and Yenugu 2019; Thoré *et al*., 2020).

Pesticide pollution is one of the main anthropogenic causes of environmental change (Thoré *et al*., 2020). The effects of pollutants are generally already well established, although some emerging substances are still poorly understood. Moreover, pollutants do not only have effects on their target species. Some pesticides, as it is often the case with neurotoxins, also impact non-target species and these actions cause problem in the environment. The stress induced by the presence of toxic substances represents a threat to ecosystems but also to our health. The DOHaD (Development Origin of Health and Disease) has shown that environmental factors can also impact our health during the fetal development of children (Blanc et al., 2020).

Nothobranchius furzeri and the aging

The species that was selected for this study is *Nothobranchius furzeri*, several characteristics make it the most interesting to use in this context.

Nothobranchius furzeri is a small teleost in the cyprinodont clade (Cellerino *et al*., 2016; Kim *et al*., 2016). It can be found in southeastern Africa, in a habitat that experiences the monsoon seasons. The dry period causes its living environment to dry out yearly, while during the wet period, the environment suffers from heavy rainfalls events (Cellerino, Valenzano and Reichard 2016). This small fish, averaging 7 cm in length, has acquired a life cycle adapted to this rhythm and is therefore present in temporary freshwater formed during the rainy period (Cellerino, Valenzano and Reichard 2016; Thoré *et al.* 2020). Its rapid life cycle allows it to follow this annual phenomenon. *N. furzeri* grows fast and reaches sexual maturity only four to five weeks after hatching (Api *et al*., 2018; Kim *et al*., 2016; Thoré *et al*., 2020). It then reproduces rapidly before its environment dries out, a female can lay between 30 and 50 eggs per day if they have access to sufficient nutrient resources (Polačik, Blažek and Reichard 2016; Thoré *et al.* 2020). All of this allows this killifishes to live an average of six months, which correlates with the length of the rainy season (Hellou, 2011; Thoré *et al*., 2020). This short lifespan is caused by a rapid age-related functional decline and change in cellular and molecular expression (Cellerino, Valenzano and Reichard 2016). The turquoise killifish is therefore the shortest-lived vertebrate (Api *et al.* 2018; Cellerino, Valenzano and Reichard 2016).

Another strategy allows this species to outlive the dry season. Its eggs are able to go into dormancy or diapause, i.e., stop their embryonic development, during the drought period, which can last about ten months (Cellerino, Valenzano and Reichard 2016; Thoré *et al.* 2020). The following figure (Fig. 10) shows the annual life cycle of *N. furzeri* (Cellerino, Valenzano and Reichard 2016). At the beginning of the year, the filling of the pools due to the arrival of the wet period causes the individuals to hatch. The eggs will then diapause in these pools for the whole time of the wet period, meaning two or three months. The eggs grow rapidly and become sexually mature in a few weeks. The sexuality can be easily differentiated because there is a very marked sexual dimorphism between them. Reproduction replenishes the egg stock, which will go dormant until the next wet period (Cellerino *et al*., 2016; Reichard & Polačik, 2019). However, not all eggs hatch in every wet episode. This allows us to remain cautious of isolated rain events. If a heavy rainfall event occurs in isolation during the dry period, the hatching of all eggs would be very dangerous for the survival of the species as the larvae will not survive the drought. The life cycle of turquoise killifish is perfectly suited to their habitat, which can be ephemeral and unpredictable (Cellerino *et al*., 2016; Reichard & Polačik, 2019).

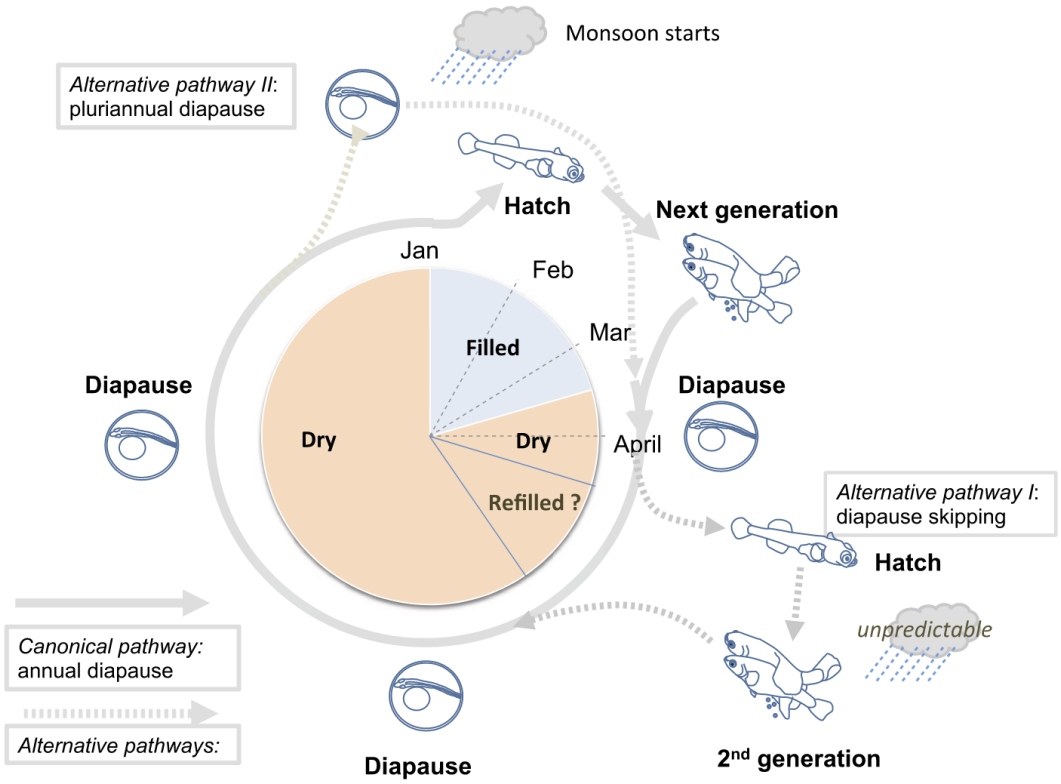


Figure 10: Diagram of the life cycle of Nothobranchius furzeri (Cellerino, Valenzano and Reichard 2016).

This process appears to be fairly fixed, but environmental fluctuations can still interact with sexual maturity, reproduction or growth of individuals. The plasticity of this species allows it to differentially allocate its energy to reproduction and egg production or to growth when conditions are not good. Once conditions become better, a compensatory increase in growth can also occur (Cellerino *et al*., 2016; Kim *et al*., 2016). This flexibility is very interesting for a species living in such a changing environment.

Different studies have shown that the maximum lifespan of turquoise killifish is an intrinsic factor of the species that varies little in captivity (Cellerino, Valenzano and Reichard 2016; Polačik, Blažek and Reichard 2016). Indeed, it remains correlated with the duration of the wet period in the area where each sample was collected. Thus, it can vary around 12 weeks in semi-arid areas while in sub-humid regions it can reach 45 weeks (Graf, Cellerino and Englert 2010).

N. furzeri is a key organism in the study of aging and is used in long-term studies because of its time and cost savings (Reichard & Polačik, 2019; Thoré *et al*., 2020). The potential of this species as a model organism goes even further. Its characteristics allow a study of aging and associated long-term dysfunctions at the biological, molecular and reproductive levels. Moreover, they are very close to those of human development. This is why *N. furzeri* is an emerging model in the ecotoxicological study of behavior (Api *et al.* 2018; Cellerino, Valenzano and Reichard 2016; Hellou 2011; Thoré *et al.* 2020).

Molecular level

Molecular analysis allows us to learn more about the expression of genes of interest. The level of expression is studied in order to highlight a difference between fish that have been exposed differently to the environmental conditions imposed. The presence of permethrin and parental age could play a role in gene expression. The *N. furzeri* genome is already well known, reaching a size of 1.3-2.2 Gb and having 28,494 protein-coding genes, of which 6,000 have already been identified (Khan *et al*., 2019). To analyze how gene expression is affected, the genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), IGF-2 (insulin-like growth factor-2), keap1b (Kelch-like ECH-associated protein 1), mTOR (mechanistic Target Of Rapamycin), SIRT1 (sirtuin 1), SIRT2 (sirtuin 2), PSMA1 (proteasome type 1 alpha subunit), PSMB1 (proteasome type 1 beta subunit), NEF2 (nuclear factor E2 related factor 2) would be interesting to analyze for their involvement in different processes.

The different mechanisms studied are related to oxidative stress and aging. Keap1b is involved in the protection of cells from oxidative stress and electrophiles (Nguyen *et al*., 2020). Keap1b in fish and amphibians corresponds phylogenetically to keap1 in mammals (Nguyen *et al*., 2020). It acts as a stress sensor and inhibitor of NEF2, a transcription factor linked to the expression of genes involved in cellular and oxidative protection (Kloska *et al*., 2019; Nguyen *et al*., 2020). In rodents its involvement in senescence has also shown a correlation with lifespan (Kloska *et al*., 2019).

Many genes of interest are also involved in growth, metabolism and senescence. GAPDH acts primarily at the level of the nucleus, both in DNA repair, autophagy and cell death (Udroiu *et al*., 2022). The mechanisms are still poorly understood but translocation into the nucleus would be linked to the presence of environmental stress (Udroiu *et al*., 2022). SIRT1 and SIRT2 are also involved in apoptosis and DNA repair. Sirtuins are also involved in the control of lipid metabolism, mitochondrial respiration and aging (Arif *et al*., 2022). Overexpression of SIRT1 could be responsible for the increase in antioxidant activity (Arif *et al*., 2022). It is able to bind to the transcription factor NEF2 (Singh & Ubaid, 2020). mTOR and IGF-2 are also involved in growth regulation (Husak & Lailvaux, 2022). Their association is important in the switch between anabolic and catabolic processes. IGF-2 is directly involved in energetic allocation and the survival-reproduction trade-off (Husak & Lailvaux, 2022). mTOR is known to integrate environmental factors such as growth factor signals or nutritional status (Liu & Sabatini, 2020). Their roles are therefore closely linked. Finally, PSMA1 and PSMB1 are enzymes that have an important role in the degradation of intracellular proteins (Yang *et al*., 2022). They regulate DNA repair, cell cycle progression and apoptosis (Yang *et al*., 2022). In general, the presence of PSMB1 is correlated with reduced overall survival (Guo *et al*., 2022).

2. Additional protocols

Appendix 1: Breeding protocol for *Nothobranchius furzeri*

The purpose of this protocol is to explain in detail the breeding process of *Nothobranchius furzeri*.

I. Preparation of the coconut fiber

This step is to be done before the harvest of the eggs. A block of dry coco fiber is rehydrated to saturation with distilled water and stored in a dark and cold place.

For the preparation of the coco boxes, a quantity of coconut fiber is well wrung out before placing it in the Petri dish. It is necessary to have about 1 cm of coir in the dish. It is then compacted and sterilized using a press disinfected with alcohol and passed under the flame of a Bunsen burner. This operation can be repeated 2 or 3 times. The boxes are then closed with parafilm to prevent them from drying out while waiting to lay the eggs.

II. Preparation of the breeding tanks

This operation is carried out the first day of reproduction. Then, the tanks will be reused from day to day. The breeding tanks are filled with about 1 cm of fine sand able to pass through the filter mesh (0,7 mm).

A sandbox is placed gently in the breeding tanks before each breeding event.

III. Collection of the eggs

The breeding tanks filled with sand are taken out of the breeding aquariums. The water is partially emptied and the tanks are identified to their respective aquarium. The contents of the tanks are then filtered through a 0.7 mm filter that allows the sand grains to pass. This allows to keep the eggs which are bigger (1 mm). The sand is collected in another breeding tank to be reused immediately.

The eggs are collected with a transfer pipette and placed in a Petri dish containing a bottom of water from the system. Then they are examined under the microscope in order to remove those which seem not to have been fertilized or which are dead. The microscope allows

to identify eggs with abnormalities synonymous with the absence of fertilization or the death of the egg. A healthy fertilized egg has 2 membranes (1 chorion and 1 fertilization membrane), it is well rounded with lipid droplets. The embryo is visible as a cluster of 2 to several cells depending on the time of development. This cluster seems to be inserted between the two membranes. The NIS-element-bis program allows to observe the eggs on the computer.

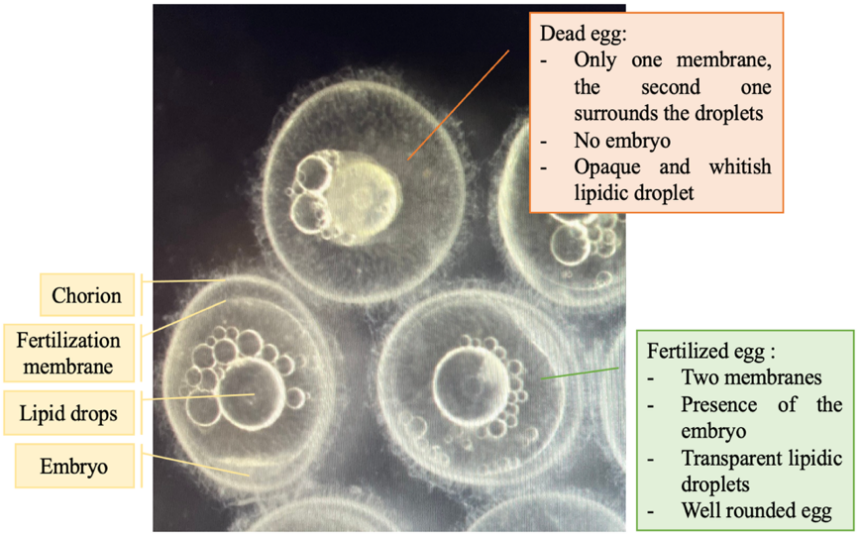


Figure 12: Photograph of a fertilized (green) and unfertilized (red) egg and the different tissues to identify in the fertilized egg (yellow).

IV. Bleaching

This step allows to clean the eggs as much as possible before their incubation to prevent the development of fungus or other impurities which could affect their development. It is therefore especially important to realize when the eggs are intended to hatch.

This cleaning consists in passing the eggs in 2 baths of H_2O_2 and 3 baths of methylene blue. First of all, it is necessary to prepare the two solutions with the expected concentrations:

- H_2O_2 at 0.5 % (2 mL of H_2O_2 at 30 % in 120 mL of system water)
- Methylene blue at 1 % (200 μL in 2 L of system water)

H_2O_2 is very sensitive, so its preparation should be done at the time of bleaching to avoid its degradation by light. When the solutions are ready, the eggs are moved to a filter which is soaked successively in each bath for about 30 seconds. The first one is H_2O_2, then the water system and finally the methylene blue. At this stage, the eggs are moved to a new box containing methylene blue to be kept at 28°C until their development.

V. Transfer of the eggs on the coconut

When the coconut box is sterilized and the eggs are cleaned, it is necessary to transfer the eggs on the coconut. For this, a flexible clamp is used to avoid damaging the eggs. The eggs must be sufficiently spread out so that the fungus does not spread if an egg dies. There can be about 100 or 150 eggs at most in a box.

When all the eggs are placed on the coco, the box is closed and sealed with parafilm. This step must be done delicately to avoid that the eggs roll in the box and stick.

Finally, the box is annotated and placed in an incubator at 28°C to allow development or at 17°C to induce diapause.

VI. Golden eyes stade

At the end of their development, after about 4 weeks, the embryos reach a stage where their golden eyes are visible through the egg. This stage is the sign that they are ready to hatch, it is called the "golden eyes" stage.

Appendix 2: RNA and DNA extraction adapted protocol with the Quick-DNA/RNA Miniprep Plus kit (#D7003) from Zymo Research

MATERIAL:

- Kit Quick-DNA/RNA™ Miniprep Plus kit
- Ethanol 100 %
- Nuclease-free water
- Nuclease-free tubes

I. Buffer preparation

1. Add 96 mL of 100 % ethanol to the 24 mL DNA/RNA Wash Buffer concentrate
2. Reconstitute lyophilized Proteinase K at 20 mg/mL with 1.04 mL Proteinase K Storage Buffer and mix by vortexing. Use immediately or store frozen aliquots.
3. To prepare a 1X solution, add an equal volume of nuclease-free water (not provided) to DNA/RNA Shield (2X concentrate) and mix well.

II. Sample preparation

Perform all steps at room temperature and centrifugation at 10 000 g for 30 seconds.

1. Eggs are homogenized directly in 100 μL DNA/RNA Shield (1X) manually and with 0.5 mm bead beating during 5 min at a power of 12 in the bullet blender.

2. Centrifuge to remove bubbles. Add 200 μL DNA/RNA Shield (1X) to obtain a total of 300 μL.

3. In the 300 μL sample with beads, add 30 μL PK Digestion Buffer and 15 μL Proteinase K.

4. Mix and spin and then incubate at 55°C for 30 minutes at 400 rpm.

5. To remove particulate debris and beads from homogenized tissue, centrifuge and transfer 250 μL of the cleared supernatant into a nuclease-free tube.

6. Add 345 DNA/RNA Lysis Buffer to the supernatant (1:1) and mix well and spin.

III. DNA and RNA purification

Perform all steps at room temperature and centrifugation at 12 000 g for 1 min, unless specified.

1. Transfer the sample into a Zymo-Spin IC-XM Column in a Collection tube and centrifuge.

2.

DNA Purification	RNA Purification
2.1. Transfer the Zymo-Spin IC-XM Column into a new Collection tube	2.2. Add 700 μL ethanol 100% to the flow-through (1:1) and mix well (up and down) Then, transfer the sample (max 700 μL at a time) into a Zymo-Spin IC Column in a Collection tube and centrifuge. Discard the flow-through.

3. Add 400 μL DNA/RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

4. Add 700 μL DNA/RNA Wash Buffer to the column and centrifuge. Discard the flow through.

5. Add 400 μL DNA/RNA Wash Buffer to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer. Then carefully, transfer the column into a nuclease-free tube (not provided).

6. Add 70 μL DNase/RNase-free Water directly to the Zymo-Spin IC-XM Column and 30 μL to the Zymo-Spin IC Column, let stand for 5 minutes and then centrifuge 2 minutes at 12 000 g and 4°C.

7. The 70/30 μL eluted DNA/RNA can be used immediately or stored frozen.

Appendix 3: Evaluation of the amount of DNA and RNA on nanodrop

Nanodrops are useful to know the concentration of DNA/RNA in a sample. It also allows to highlight a possible contamination thanks to a reading at different wavelengths.

- the absorbance at 260 nm shows the presence of nucleic acids.
- the absorbance at 280 nm shows the presence of tryptophan thus proteins.
- the absorbance at 230 nm shows the presence of solvent (ethanol).

The ratio 260/280 thus informs of a contamination in proteins in the sample. Ideally, it should be between 1.8 and 2.2.

The ratio 260/230 indicates a solvent contamination in the sample. Ideally, it should be between 1.8 and 2.2.

Appendix 4: Verification of the extraction by electrophoresis on Agarose gel

1. Using a graduated cylinder, measure the volume of 0.5X TAE needed (20 mL for a small and 40 mL for a large) and transfer it to an Erlenmeyer flask.

2. Measure the quantity of agarose powder necessary to obtain the gel with the desired percentage and mix it with the TAE (0.3 g of powder for a small gel of 1.5 %).

3. Boil the solution of TAE and agarose in the microwave.

4. Transfer the solution to a contaminated tube (at Syber Safe) and wait for it to cool slightly.

5. Add 0.5 μL of Cyber Safe per 10 mL of 0.5X TAE buffer, mix gently and pour the solution into the rack containing the caster and comb. Protect from light and let polymerize for about 45 minutes.

6. Once polymerization is complete, carefully remove the comb and place the caster containing the gel in the electrophoresis tank. Check that the TAE level in the tank covers the gel.

7. Load the wells with the samples to be tested. Beforehand, 1 μL of Loading Dye is mixed by up and down with 4 μL of each sample. In order to avoid bubbles when loading the gel, 4 μL of this mixture will be placed in each well.

8. Start the electrophoresis at 100 Volts for 45 min.

9. Once the electrophoresis is finished, visualize the gel with the ChemiDoc® system program.

Appendix 5: DNase treatment protocol with the DNA-freeTM Kit_DNase Treatment and Removal Reagents (#AM1906) from ThermoFischer

- We recommend conducting reactions in 0.5 mL tubes to facilitate removal of the supernatant after treatment with the DNase Inactivation Reagent.
- DNA-*free*™ reactions can be conducted in 96-well plates. We recommend using Vbottom plates because their shape makes it easier to remove the RNA from the pelleted DNase Inactivation Reagent at the end of the procedure.
- The recommended reaction size is 25 µL. The protocol below is adapted to a reaction size of 25µl.
- There are separate DNase digestion conditions depending on the amount of contaminating DNA and the nucleic acid concentration of the sample. If you suspect significant DNA contamination DNA (i.e., >1 µg DNA/25 µL), refer to the kit protocol.

1. Pipette up to 5 µg of totRNA into a sterile nuclease-free tube on ice (max 21.5 µl), then bring the volume to 21.5 µl with nuclease-free water. Add 2.5 µl of 10X DNase I Buffer and 1 µL rDNase I to your sample, and mix gently

2. Incubate at 37°C for 20–30 min.

3. Add 2.5 µl resuspended DNase Inactivation Reagent and mix well. Always resuspend the DNase. Inactivation Reagent by flicking or vortexing the tube before dispensing it.

Note: The DNase Inactivation Reagent may become difficult to pipette after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Nuclease-free Water (supplied with the kit) equal to approximately 20–25% of the bed volume of the remaining DNase Inactivation Reagent, and vortex thoroughly to recreate a pipettable slurry.

4. Incubate 2 min at room temperature, mixing occasionally. It is important to mix the contents of the tube 2–3 times during the incubation period to redisperse the DNase Inactivation Reagent.

5. Centrifuge at 10,000 × g for 1.5 min and transfer the RNA to a fresh tube. For 96-well plates, centrifuge at 2000 × g for 5 min. This centrifugation step pellets the DNase Inactivation Reagent. After centrifuging, carefully transfer the supernatant, which contains the RNA, into a fresh tube. Avoid introducing the DNase Inactivation Reagent into solutions that may be used for downstream enzymatic reactions, because it can sequester divalent cations and change the buffer conditions.

Appendix 6: Reverse Transcription protocol with Thermo Scientific RevertAid RT Kit (#K1691 500 rxns) from ThermoFischer

After thawing, mix and briefly centrifuge the components of the kit store on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	
Total RNA	0.1 ng – 5 μg
Random Hexamer primer	1 μL
Water, Nuclease-free	To 12 μL
Total volume	12 μL

2. Mix gently, centrifuge briefly and incubate at 65 °C for 5 min. Chill on ice, spin down and place the vial back on ice.

3. Add the following components in the indicated order*:

Reagents	**Final volume for 1 reaction**
5X Reaction Buffer	4 μL
RiboLock RNase Inhibitor	1 μL
10 mM dNTP Mix	2 μL
RevertAid RT (200 U/μL)	1 μL
Total volume	20 μL

*A mastermix (n+2) can be prepared on ice few moments before use (max 10min). Add 8 µl of the mastermix to the 12 µl of sample and primers.

4. Mix gently and centrifuge briefly.

5. For random hexamer primed synthesis, incubate for 5 min at 25 °C followed by 60 min at 42 °C.

Note. For GC-rich RNA templates the reaction temperature can be increased up to 45 °C.

6. Terminate the reaction by heating at 70 °C for 5 min.

The reverse transcription reaction product can be directly used in PCR applications or stored at -20 °C for less than one week. For longer storage, -70 °C is recommended.

Appendix 7: Verification of DNase and RT treatments by PCR (Polymerase Chain Reaction) amplification with the GoTaq® G2 DNA Polymerase (# M7841)

I. PCR mix

Component	**Final volume of 1 reaction**	**Final concentration**
Upstream/Downstream primer 2.5 µM	2.5 µL	0.25 µM each primer
Template DNA	1 µL	< 0.25 µg/25 µL
5X Green or Colorless GoTaq® Reaction Buffer*	5 µL	1X (1.5 mM $MgCl_2$)
PCR Nucleotide Mix, 10 mM each (Cat. #C1141)*	0.5 µL	0.2 mM each dNTP
Nuclease-Free Water*	1.9 µL	
GoTaq® G2 DNA Polymerase (5 u/µL)*	0.12 µL	0.6 u
Total volume	25 µL	

*A master mix can be made with these components and 21.5 µL is deposited in each well already containing the Upstream/Downstream primer and Template DNA.

Notes: GoTaq® G2 DNA Polymerase should be added last because this enzyme is very sensitive.

II. General guidelines for Amplification by PCR

Step	Temperature	Time	
Initial denaturation	95 °C	2 minutes	
Denaturation	95 °C	0.25 – 1 minute	25 – 35 cycles
Annealing	42-65 °C	0.25 – 1 minute	
Extension	72 °C	0.5 – 2 minutes	
Final extension	72 °C	5 minutes	
Soak	4 °C to 10 °C	Infinite	

Notes:

- Temperature approximatively 5 °C below the Tm of the primers (optimization needed) 42 – 65 °C.
- One minute for 1 kb of DNA to be amplified. The time of the cycles depends on the size of the fragments to be amplified.

Appendix 8: Quantitative Polymerase Chain Reaction (qPCR) analysis

I. Preparation of negative controls

Two negative controls allow to check the good functioning of the analysis. The first one verifies that the DNase has worked properly and that the samples do not contain any DNA.

1 μL of each sample is taken after the DNase treatment and before the RT. A pool is created with all these samples (48 μL for 48 samples) and 200μL of Nuclease-Free Water is added to have enough volume for the analysis of all the genes to test.

The second control contains only Nuclease-Free Water to show possible contamination of the plate. It is called NTC (No Template Control).

II. Preparation of the standards

The standards allow to check the efficiency of the primers (around 100%) on the samples to be tested. A pool is created with 20 μL of each post-RT sample diluted 5X, this represents the first standard (STD1). A serial dilution from 4 to 4 is then performed for the other standards.

200 μL of STD1 is added to 600 μL of nuclease-free water and so on for the other standards. The standards obtained are then diluted 5, 20, 80, 320, 1280X.

III. Preparation of the samples

The post-RT samples already diluted 5X are further diluted 2X into Nuclease-Free Water to reach a 10X dilution and be in the range covered by the standards.

IV. Filling of the plate

The filling of the plate for the qPCR analysis is done in 3 steps,

- 2,5 μL of the sample, standards or negative control
- 2,5 μL of the primer of the gene to be tested
- 5 μL of SYBR® Green Supermix

The plate plan is as follows (Fig. 11).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4	STD5	STD5	STD5	RT-	RT-	RT-	NTC	NTC	NTC	/	/	/
B	S1	S1	S1	S2	S2	S2	S3	S3	S3	S4	S4	S4	S5	S5	S5	…								
C	S9	S9	S9	S10	S10	S10	S11	S11	S11	S12	…													
D	S17	S17	S17	S18	S18	S18	…																	
…	…																							

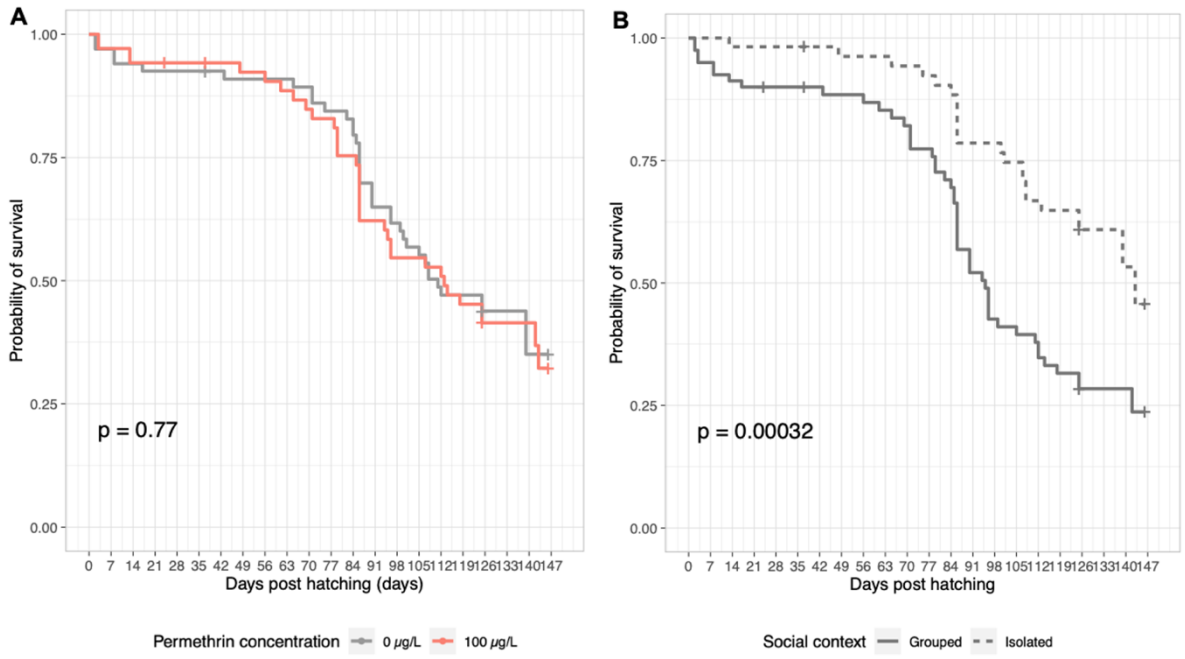
Figure 11: STD = standard, S = sample.

3. Additional data

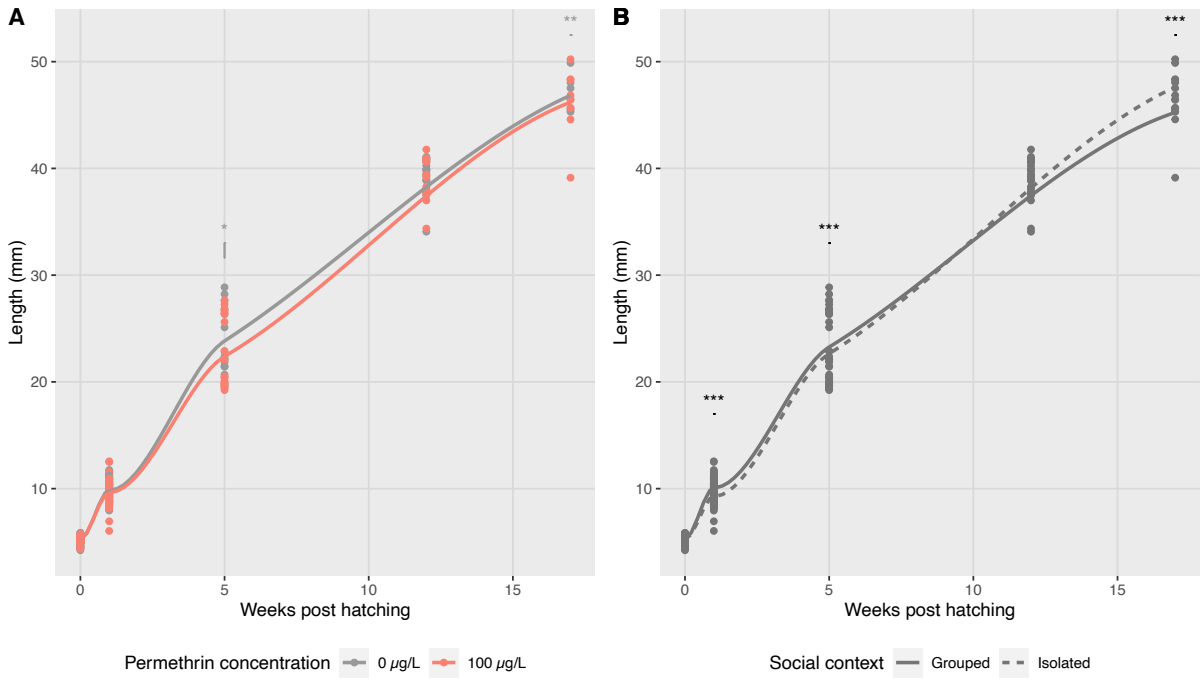
Appendix 9: distribution of individuals in the different conditions.

Social context	**Community group**		**Isolated group**	
Permethrin concentration	Control (CTL)	Permethrin (C1)	Control (CTL)	Permethrin (C1)
Cohort 1	N = 10	N = 10	N = 9	N = 9
Cohort 2	N = 30	N = 30	N = 18	N = 20
Total	N = 40	N = 40	N = 27	N = 29

Appendix 10: A. Kaplan Meier survival curve according to the permethrin concentration. B. Kaplan Meier survival curve according to the social context.



Appendix 11: Evolution of the height over the whole life.



A: Evolution of the size according to the permethrin concentration during the exposure. B: Evolution of the size according to the social context during the exposure.

Appendix 12: Summary table of primers for the genes studied in this study

The genes in green represent those that were selected for further analysis. In red, the preliminary tests on the primers were not good enough to perform the qPCR. The PSMA1 primer was not very specific, many samples showed several metling temperatures. The NEF2 analysis is also unreliable because the efficiency is much higher than the others and the R2 was 0.85, which is very low. Keap1b has a rather low efficiency, so we should also be wary of it.

	Forward primer	***Reverse primer***	***Efficiency***
EF1a	GTGGAAGTTTGAGACCAGCAAG	CAACACCAGCAGCAACAATC	103.257
GAPDH	TGGTATGGCTTTCCGTGTCC	TCGTCGTACTTGGCTGGTTT	103.359
IGF2	CTCGCGCTGACTCTCACATT	CTGCTGGTTGGCCTACTGAAA	95.211
Keap1b	TCCGTTTTATCGCAGAGTCCA	TTTGCAGACGACACCAATGC	83.571
mTOR	TACAGAGCCGTTCTAGCCCT	AAGACACCATGGCCCCATAC	93.257
SIRT1	CGGGACGGAGACAACAATGA	GATCGTCATGTCCTGCGACT	103.725
SIRT2	GGACTTCCCTCGTTGTGACC	AACATGGGACCAGCCTCTCC	104.042
PSMA1	TCTTGTCGGCAGCAAAACCC	GCCAGCAATGAGGAGTCCAA	102.154
PSMB1	CGCCTTCAACGGAGGAACTG	GCAGCCTATGACAGTCGTGT	92.448
NEF2	AGGAGTGTCCATCCACGCTA	TGGGACTTGAAACTCCTGTAACT	156.647

Appendix 13: Summary of the means and standard deviations of the expression of the genes of interest in function of the exposure concentration and at different time-points.

Permethrin concentration	Parental age	GAPDH	IGF-2	mTOR	SIRT1	SIRT2	PSMB1
0 g/L	8 weeks	Mean = 1.34 Sd = 1.09	Mean = 1.13 Sd = 0.57	Mean = 1.30 Sd = 0.84	Mean = 1.03 Sd = 0.24	Mean = 0.97 Sd = 0.31	Mean = 1.51 Sd = 1.12
100 μg/L	8 weeks	Mean = 1.51 Sd = 1.09	Mean = 1.35 Sd = 0.55	Mean = 1.45 Sd = 0.78	Mean = 1.12 Sd = 0.50	Mean = 1.05 Sd = 0.30	Mean = 1.62 Sd = 1.07
0 g/L	12 weeks	Mean = 1.24 Sd = 0.79	Mean = 1.15 Sd = 0.60	Mean = 1.06 Sd = 0.39	Mean = 1.05 Sd = 0.32	Mean = 1.02 Sd = 0.21	Mean = 1.28 Sd = 0.85
100 μg/L	12 weeks	Mean = 1.13 Sd = 0.86	Mean = 1.00 Sd = 0.46	Mean = 0.92 Sd = 0.22	Mean = 0.93 Sd = 0.28	Mean = 1.09 Sd = 0.25	Mean = 1.02 Sd = 0.76
0 g/L	15 weeks	Mean = 1.02 Sd = 0.25	Mean = 1.05 Sd = 0.34	Mean = 1.03 Sd = 0.27	Mean = 1.06 Sd = 0.38	Mean = 1.04 Sd = 0.22	Mean = 1.077 Sd = 0.46
100 μg/L	15 weeks	Mean = 0.84 Sd = 0.26	Mean = 1.15 Sd = 0.47	Mean = 1.07 Sd = 0.47	Mean = 0.82 Sd = 0.38	Mean = 0.75 Sd = 0.23	Mean = 0.84 Sd = 0.49

Appendix 14: Experimental design