A model for ageing

August 7 2015

With its red and black bands and black-yellow patterned fins, the Turquoise killifish doesn't really look turquoise. It gets its name from the greenish-bluish basic colour of its scales. After only a few months, the shining colours of the young fish begin to fade. The rapid physical decline of the fish has aroused the interest of age researchers around the world. Credit: Frank Vinken

Life is short, especially for the killifish, Nothobranchius furzeri: It lives for only a few months and then its time is up. During that short lifespan it passes through every phase of life from larva to venerable old fish. Its brief life expectancy – unusual for a vertebrate – has long fascinated Dario Valenzano of the Max Planck Institute for Biology of Ageing in Cologne. During a ten-year period he has made it a model organism for research into ageing.

"Wow, that's a really old fish!" Valenzano can barely hide his astonishment in the fish basement of the Max Planck Institute in Cologne. Aquariums are lined up end to end on long shelves.

Who, however, was expecting to find a date of birth in the distant past, will be surprised to read September 2014 on the birth certificate stuck on the aquarium pane. The presumed Methuselah was just nine months old on the day of the visit to the fish basement. For a member of this Nothobranchius species, a positively biblical age. No other vertebrate ages so swiftly.

Valenzano encountered the fish – named after its discoverer, Richard Furzer – in 2002, as a student in the laboratory of his mentor Alessandro Cellerino in Pisa. The laboratory had a small aquarium stocked with fish. Cellerino had been given the fish by an acquaintance, a hobby aquarist who had bred killifish for many years. The fish buff drew the two researchers' attention to the phenomenal rate at which the fish ages.

The young student had no specific interest in fish at the time. He was more focused on the behavior and evolution of apes and humans. For his Master's thesis work, he studied apes in a zoo for months and analyzed their facial expressions. Nevertheless, the ephemeral fish caught his interest and soon became his passion. Out of curiosity, he looked for signs of ageing in the brains of the fish and found the same protein deposits that are typical of ageing in the human brain.

From then on, Valenzano wanted to solve the puzzle of the short lifespan of Nothobranchius and make it a model organism for research into ageing. Several models already existed: for example, the nematode C. elegans, the fruit fly Drosophila and the mouse. The latter lives for two to three years. That may not sound like much, but that's also how long researchers have to wait before they can examine a mouse in advanced age. Nematodes and fruit flies live for just a few weeks, but, as non-

vertebrates, they significantly differ from humans. "I wanted to develop the vertebrate equivalent of Drosophila to study the biology of vertebrate aging," explains Valenzano.

Nothobranchius would therefore fill a gap: It has an extremely short lifespan and, as a vertebrate, it is closely related to humans. In just a few months, Nothobranchius undergoes the entire ageing process that takes years or decades in other vertebrates. Why has this fish in particular not been blessed with a long life? After all, some fish become very old. Koi carp, for example, can live for several decades. One species of rockfish in the North Pacific even has a life span of over 200 years.

The short lifespan of the killifish could be correlated to the climate of its habitat in southern Africa, the home of the turquoise killifish, how it is also called. Water is present there for seven months of the year at most; some bodies of water dry up completely after just two months. This is probably not the most auspicious situation for fish longevity. Only fish that are able to develop and reproduce while there is still water available are able to survive. On the other hand, there is no gain for the fish in being longer-lived. Therefore, selection will not favor genes that make it live beyond the point where the water in the puddles dries out. For Valenzano, this could be the key to understanding how a short lifespan evolved in this species.

Due to the short rainy season, Nothobranchius furzeri has evolved to mature quickly: The fish grow into adults capable of reproduction just three to four weeks after hatching. Then, the shimmering colors of youth pale, the fins fray, and the spine becomes progressively curved. The fish passes through every ageing phase, including dotage, rapidly, as in a time-lapse film.

Nature seems indifferent to this, as long as the survival of the next fish generation is ensured. The eggs develop at the bottom of the pond. If the

pond dries up, the embryos fall into a state of suspended animation and can survive months of drought.

In advanced age Nothobranchius is rather inflexible. It ages, even when there is no threat of early death due to desiccation. That's what fascinated Valenzano so much about the fish: "Nothobranchius could give us an answer to the question of why ageing occurs at all. Does ageing confer an advantage on plants and animals? Or is there simply no reason to prevent the inevitable process of decay, once successful reproduction has taken place?"

Although ageing cannot be halted in Nothobranchius, it can be slowed. A number of factors influence the life expectancy of the fish, one being temperature: the cooler the water, the more slowly the fish age. Food supply also plays a role. If there is a scarcity of food, the fish live longer – a phenomenon that researchers have also observed in fruit flies and nematodes. Why this is so has not been fully explained. "Perhaps the temperature and food supply give the fish cues as to whether the environmental conditions are favorable. At low temperatures and when food is scarce, it makes sense to wait awhile before reproducing. Consequently, the fish must stay alive longer to reproduce," Valenzano suspects.

But the life history of the killifish is not only interesting in itself. To Valenzano, it also offers an opportunity to understand how other organisms age, including humans. Ageing killifish develop cancer, show cognitive decline, become less fertile, lose their pigmentation and become more fragile. These are very general ageing phenotypes, shared by many organisms. "Nothobranchius allows us to study in a short time how these biological mechanisms work," explains Valenzano.

Many characteristics have to come together for a species to serve as a model organism for science. Nothobranchius furzeri is a success story in

this respect. Valenzano has elevated it to an object of global scientific interest. Some 40 laboratories around the world are now working with this species. "Every fortnight or so we receive enquiries from scientists to send them Nothobranchius eggs so that they can breed the fish in their laboratory," Valenzano says. Now, there is even an international scientific conference on the turquoise killifish held every two years.

When scientists have to improvise ... As a basis for the genetic and gut analyses in the Savannah, Valenzano and his colleagues use a simple folding table - and an ironing board. Credit: MPI for the Biology of Ageing – Dario Valenzano

Worldwide Killifish boom

The road leading to this point was rocky, and Valenzano's plans did not always evoke enthusiasm. Some members of his PhD thesis committee, before he moved to Stanford University for his postdoc, were cautious about the ambitious project, warning him about the risk of developing something new. Nevertheless, they all supported his project and provided him with the opportunity to establish genetic tools to study ageing in Nothobranchius.

First, he investigated whether Nothobranchius simply dies young without first ageing appreciably. He also wanted to shed light on precisely how the fish ages. He discovered protein deposits and damage in the brain that became increasingly common with advancing age. He also found learning deficits: Older killifish learn less quickly to associate a harmless light stimulus with a frightening mechanical disturbance in the water. As in humans, ageing affects multiple organs in killifish. The animals become more sluggish and lose weight. Also, their spine begins to contort. The kidneys become less efficient, and tumors grow in the liver. "Cancer is the most common cause of death in laboratory killifish," Valenzano says.

He also had to draw up instructions for keeping and breeding the fish. Although Nothobranchius is undemanding and quite easy to keep, the fish must live under similar conditions to allow the findings from different research laboratories to be compared. Valenzano therefore developed protocols detailing the chemical composition of the water, temperature, light and food. The ageing process, after all, is affected by numerous environmental factors.

To serve as a model for ageing research, an animal must have a sequenced genome that allows the scientific community to study specific genes and design strategies to manipulate them. Valenzano has therefore taken great efforts to develop suitable molecular biological methods, for example the transfer of DNA to Nothobranchius eggs. The eggs are

covered by a tough outer sheath to prevent them from drying out. "At first, the sheath caused us a lot of headaches. We were unable to penetrate it to inject genes using conventional microneedles. We weren't successful until we used shorter, harder needles and a few other tricks, mostly discovered by trial and error" Valenzano explains.

Through his needle, he injected a "jumping gene" into the eggs. The gene produces an enzyme that snips the genome at specific places. In this way, Valenzano introduced a foreign gene into the genome of Nothobranchius furzeri for the first time. Without proof that Nothobranchius can be genetically modified, its model career would have been a non-starter. In the process, Valenzano discovered chromosome segments that control ageing in the killifish. He plans to study these regions in detail to determine which genes are responsible. He has also identified genes for the color of the fish's tail and others that determine the sex of the fish.

In the meantime, Valenzano and others have built up a set of tools which allow them to analyze the killifish genome as precisely as that of fruit flies and mice. Recently, the entire genome of Nothobranchius furzeri was decoded. Its sequence will be available to the scientific community soon.

Scientists are now even able to switch off Nothobranchius genes using the CRISPR/Cas9 method. With the help of this technique, which has recently revolutionized biological sciences, US researchers were able – in the space of just two to three months – to breed genetically modified Nothobranchius strains that show typical signs of ageing, such as reduced fertility and susceptibility to tumors at the tender age of just two months. The trigger for these changes is a dysfunctional gene for the telomerase protein. This enzyme normally prevents the end caps of chromosomes, the telomeres, from becoming progressively shorter over time. Shortened telomeres also occur in humans in advanced age.

But Valenzano is not only interested in the genome. He hypothesizes that the intestinal flora holds another key to understanding the ageing process of many organisms, including the killifish. As in humans and most other animals, myriad bacteria help their host to digest food and contribute to metabolic processes, which influence predispositions to important diseases such as diabetes. Every fish species has its own resident bacterial community, which can even differ from fish to fish.

Valenzano characterized which bacteria occur in the fish's intestine by analyzing the genomes of the microorganisms. "We now know that older fish have different intestinal flora than young fish, more associated to pathological states," Valenzano says. As a next step, he hopes to determine whether fish that live particularly long have different microorganisms than their short-lived counterparts, and whether he can prolong the lifespan of a fish by altering its intestinal flora. To do so, he first purges the intestinal flora with an antibiotic and then transplants microbes from a young into an old fish by adding the latter's intestinal content to the aquarium water. In this way, he wants to see whether he can manipulate fish longevity and health status.

In future, Valenzano, together with colleagues in his Research Group at the Max Planck Institute, wants to take the experiments out of the laboratory. He is bent on studying the fish in the wild. Evolution has spent millions of years devising ways to help animals cope successfully in habitats with fluctuating environmental conditions. Nothobranchius furzeri has made a virtue out of necessity: it simply lives fast and dies early.

Other fish do not sacrifice their longevity. Lungfish, for example, which live in the same ponds as Nothobranchius, burrow deep into the mud, where they wait for the drought to end. Some lungfish can reach the ripe old age of 50 years or more. Related species of Nothobranchius in the New World have solved the problem quite differently. North American

killifish jump out of drying ponds and survive the dry period on land in damp wood.

"Evolution is one big experiment in which gene variants are constantly being tested and the most suitable are selected," says Valenzano. He hopes that these naturally occurring variants will tell him why nature causes Nothobranchius to age rapidly and what happens in the process.

To do so, he must study fish as they occur in nature. However, until the turn of the millennium, scientific laboratories only held the progeny of the fish originally introduced by Richard Furzer. Furzer had captured the hitherto unknown Nothobranchius species in eastern Zimbabwe near the border with Mozambique in 1968 and had brought specimens to Europe. Since then, hobby aquarists have bred the offspring – and the offspring of the offspring – in their aquariums over a period corresponding to around 80 fish generations.

Because individuals of the Nothobranchius strain, known as GRZ, have only bred with individuals of the same strain during this whole time, the fish have become genetically very similar. Their genes are almost all the same variants – an ideal situation for genetic investigations. Having an inbred line is a luxury that not all model systems have. The zebrafish community, for instance, lacks a well established inbred line, which is part of the reason why it was quite hard to assemble the zebrafish genome.

This extreme inbreeding could account for the characteristic that makes these fish so interesting for science: their extremely short lifespan, even for killifish. Fish of the GRZ strain have the shortest life expectancy of all animals that can be bred in captivity: 9 weeks on average to 13 weeks at the most under controlled laboratory conditions. For a long time, it remained unclear whether the short lifespan of the GRZ strain was a consequence of decades of inbreeding or whether Nothobranchius

furzeri has a similarly short lifespan in the wild.

In 2004, Valenzano and several colleagues travelled to Mozambique in search of the killifish. Although many of the ponds have been transformed into rice paddies in recent years, the researchers found the killifish in several locations. The fish live under various climatic conditions: Regions at higher altitudes in the interior of the country are relatively dry compared to the coastal lowlands, which receives more rain. The coastal areas therefore do not dry out as quickly, and Nothobranchius has more time to develop there before finding itself stranded on dry land.

The scientists captured several dozen fish in four habitats and took them back to the laboratory, where they bred them. Now Valenzano was able to compare the life expectancy of Nothobranchius furzeri taken from the wild with that of the GRZ strain from the laboratory. He was also able to determine whether different natural climatic conditions affect the ageing process.

The wild fish live for 25 to 32 weeks, significantly longer than the GRZ fish from the laboratory. They still have a very short life expectancy for a vertebrate. The highland fish age more quickly and die sooner than the fish from the humid coastal region. In addition, harmful protein deposits accumulate more slowly in the brains of the lowland fish.

But Valenzano was still missing a piece of the puzzle: the wild relatives of the fish from which the GRZ strain originally developed. Furzer had captured the founding pair in the eastern area of Zimbabwe, a region that is even drier than the habitats in Mozambique. "The breeding conditions in the laboratory are not necessarily what make the GRZ strain so short-lived. Perhaps the extreme shortness of their lifespan is due to the extreme dryness of their natural habitat," Valenzano says.

To clarify this point, Valenzano had to journey to Gonarezhou National Park, the original home of the GRZ strain in Zimbabwe. It took five years to gather the necessary papers from the national park authorities, despite the fact that he did not plan to capture any fish but only take tissue samples for genetic and intestinal analyses. Poaching in southern Africa has escalated out of control in recent years, so that any activity in the park is carefully scrutinized, Valenzano says.

In the spring of 2015 he finally had all the necessary permits. Once again, he and a team of scientists set off on a journey to southern Africa. This time, he took an ironing board with him into the savannah. "It wasn't for our laundry, of course. What we needed was a flat surface upon which we could remove our samples. A collapsible ironing board from a hardware store was just the ticket," Valenzano remembers with a smile.

With the help of the samples from Gonarezhou, Valenzano can now compare the genome of the GRZ strain with that of the fish in Mozambique. He hopes to glean further information on which genes control the ageing process in Nothobranchius furzeri. He also plans to analyze how often the various alleles of ageing-related genes occur in nature and how that frequency changes over time. This should tell him how evolution has adapted the life expectancy of the fish to the prevailing environmental conditions. His future investigations will also focus on differences in the intestinal flora of these wild fish.

To do so, he will have to travel to Africa often. "It's a long-term project stretching over a period of 20, maybe even 30 years." That's feasible for a young scientist. His aquariums will then contain fish of the 40th or 60th generation. Translated into human lifespans, that corresponds to 1,000 to 1,500 years. Humans just can't hold a candle to the killifish as a model organism for ageing research.