

Living in Fast Motion

Life is short, especially for the turquoise killifish, *Nothobranchius furzeri*: it lives for only a few months and then its time is up. During that short life span 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. In just ten years, he has turned it into a model organism for research on aging.



With its red net-like pattern and black-and-yellow-patterned fins, the turquoise killifish doesn't really look turquoise. But the name refers to the blue-green base coloration of its scales. After just a few months, the vibrant colors of the young fish begin to fade. The fish's rapid physical decline has piqued the interest of researchers on aging worldwide.

TEXT HARALD RÖSCH

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

However, anyone expecting to find a date of birth in the distant past will be surprised to read "September 2014" on the birth certificate affixed to the aquarium pane. The presumed Methuselah was just nine months old on the day of the visit to the fish facility – a positively biblical age for a member of this *Nothobranchius* species. Most of its relatives are already dead at that age.

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. Together with his mentor, he set up a small aquarium stocked with fish. They 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 mostly focused on the behavior and evolution of primates and humans. For his master's thesis, he observed capuchin monkeys in captivity for months and analyzed their facial expressions. Nevertheless, the ephemeral fish piqued his interest and soon became his passion. Out of curiosity, he looked for signs of aging in the brains of the fish and found the same protein deposits that are typical of aging in the human brain.

From then on, Valenzano wanted to solve the puzzle of the short life span of *Nothobranchius* and make it a model organism for research into aging. Several models already existed: for example, the nematode *C. elegans*, the fruit fly *Drosophila* and the mouse. The latter lives just 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, in contrast, live just a few weeks, but as non-vertebrates, they differ significantly from humans. "I wanted to develop the vertebrate equivalent of *Drosophila*

to study the biology of vertebrate aging,” explains Valenzano.

Nothobranchius would thus fill a gap: it has an extremely short life span and, as a vertebrate, is closely related to humans. In just a few months, *Nothobranchius* undergoes the entire aging process that in other vertebrates takes years or decades. But why, of all fish, has this one not been blessed with a long life? After all, some fish live to be very old. Koi carp, for example, can live for several decades. One species of rock-fish in the North Pacific even has a life span of over 200 years.

The short life span of the killifish could be related to the climate of southern Africa, the habitat of the turquoise killifish, as it is also called. Water is present there for only a few months of the year; some bodies of water dry up completely after just two months. This probably isn't the most auspicious situation for fish longevity. Only fish that are able to develop and reproduce while water is still available are able to survive. Under such conditions, there is nothing to be gained from longevity genes, so selection doesn't favor them. For Valenzano, this could be the reason for the extremely short life span of *Nothobranchius*.

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 phase of aging, including dotage, rapidly, as if in a time-lapse film.

Nature seems indifferent to this; after all, the arrival of the next fish generation is ensured. The eggs are resting at the bottom of the pond. When the pond dries up, the embryos are safe, lying in a sort of dormant state. This allows them to survive the months of drought.

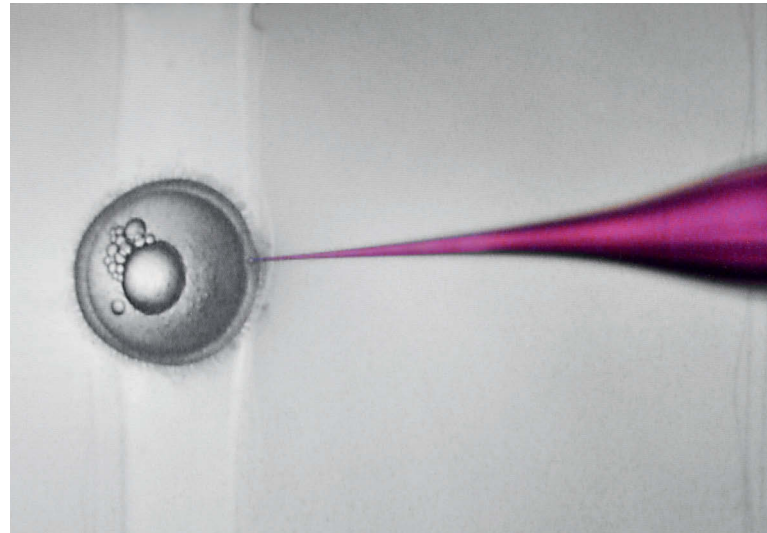
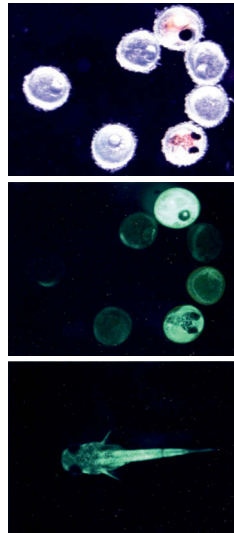
In advanced age, *Nothobranchius* is rather inflexible. It ages even when – as in the lab – there is no threat of early death due to desiccation. That's what Valenzano found so fascinating about



Photo: Frank Vinken

Left page: The key to age: Using the killifish, *Nothobranchius furzeri*, Dario Valenzano wants to find out why organisms age.

This page: Successful gene transfer: Researchers use a fine glass needle to transfer the gene for the green fluorescent protein GFP to a fertilized fish ovum (large image). In the maturing embryos (small image, top), the fluorescent gene is active (center) and causes the animals to fluoresce (bottom, five-day-old larva).



the fish: “*Nothobranchius* could provide an answer to the question of why aging occurs at all. Does aging confer an advantage on plants and animals? Or was there simply no reason to prevent the inevitable process of decay once successful reproduction has taken place?”

Although aging can’t 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 longer the fish live. 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, nematodes and rodents. Why this is so hasn’t been fully explained. “Perhaps the temperature and food supply give the fish cues as to whether the environmental conditions are favorable. When temperatures are low and food is scarce, it makes sense to wait awhile before reproducing. Consequently, the fish must stay alive longer in order to reproduce,” suspects Valenzano.

He studies these relationships with the aim of gaining a general understanding of aging. Many of the signs of aging in killifish also occur in other organisms: they develop cancer, show cognitive decline, become less fertile,

lose their pigmentation and become more fragile. “*Nothobranchius* thus allows us to study in a short time how these biological mechanisms work,” explains Valenzano.

GLOBAL KILLIFISH BOOM

Many characteristics must coincide 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 two weeks or so we receive inquiries from scientists to send them *Nothobranchius* eggs so that they can breed the fish in their labs,” Valenzano says. There is now even a biannual international scientific conference on the turquoise killifish.

The road leading to this point was rocky, and Valenzano’s project didn’t always evoke enthusiasm. Some members of his doctoral thesis committee in Pisa were cautious and warned him about the risk of developing something completely new. Nevertheless, they all supported him. Particularly Stanford University, where he went to do his postdoc work, gave him the opportuni-

ty to develop genetic tools to study aging in killifish.

First, he investigated whether *Nothobranchius* simply dies young without first aging 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, aging affects multiple organs in killifish. The animals become more sluggish and lose weight. Also, their spine begins to curve. 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 labs to be compared. Valenzano therefore developed detailed protocols for the chemical composition of the water, and for temperature, light

» Does aging confer an advantage, or was there simply no reason for evolution to prevent the inevitable process of decay once successful reproduction has taken place?



Male killifish at five (top) and twelve weeks of age (bottom). The colors fade with age, and the fins become frayed. Since the eyes don't stop growing, older fish also have larger eyes.

and food. After all, the aging process is affected by numerous environmental factors.

To serve as a model for aging research, an animal must have a sequenced genome that allows researchers to study specific genes and design strategies to manipulate them. Valenzano has therefore gone to great lengths 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 locations. 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 wouldn't have made it out of the starting blocks. In the process, Valenzano discovered chromosome segments that control aging in the killifish. He plans to study these regions in detail to determine which genes are responsible. He 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 that 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 soon be available to the scientific community.

Scientists are now even able to switch off *Nothobranchius* genes using the CRISPR/Cas9 method. Using this technique, which has recently revolutionized the 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 aging, 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 progres-

sively shorter over time. Shortened telomeres also occur in humans in advanced age.

But Valenzano is interested in more than just the killifish genome. He hypothesizes that the intestinal flora holds another key to understanding the aging process of many organisms, including the killifish. As in humans and most other animals, myriad bacteria help their host digest food and contribute to metabolic processes that influence predispositions to such diseases as diabetes. Every fish species has its own resident bacterial community, the composition of which can even differ from one fish to another within a species.

MICROBES IN FISH INTESTINES

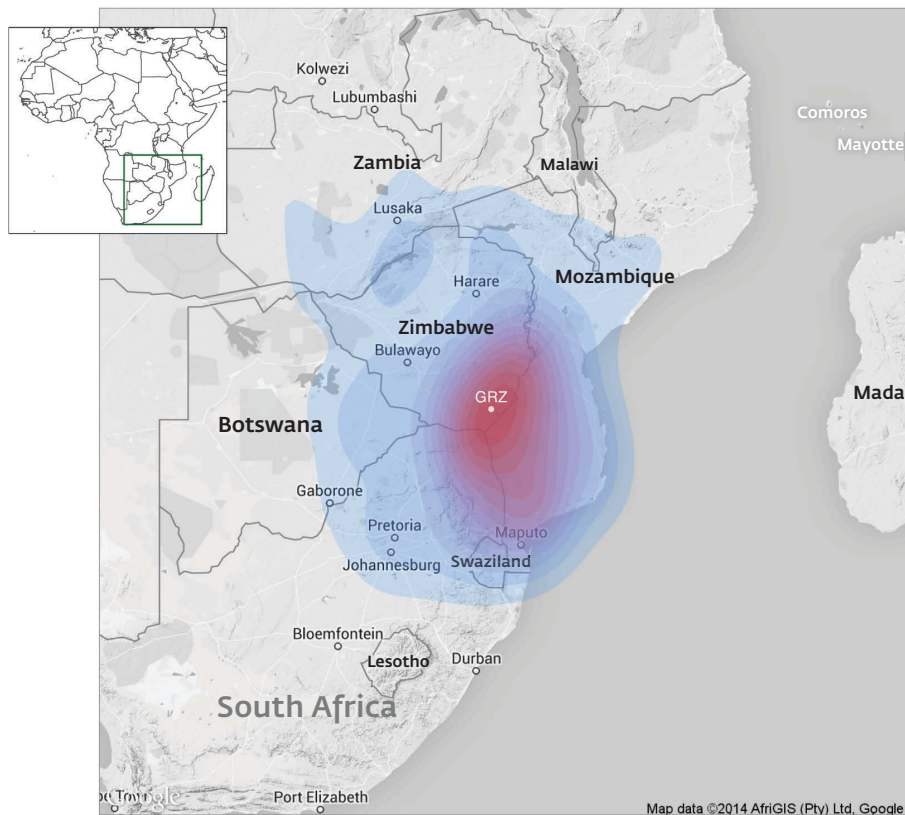
Valenzano characterized which bacteria occur in the fish intestines by analyzing the genomes of the microorganisms. "We now know that older fish have different intestinal flora than younger fish, more associated with pathological states," Valenzano says. As a next step, he wants to determine whether fish that live to be particularly old have different microorganisms than their short-lived counterparts, and whether he can prolong the life span 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 an old fish by adding its intestinal content to the aquarium water. In this way, he can study whether the old fish can live longer and remain healthy with the bacteria from young fish intestines.

In the 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. After all, 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 faster and dies sooner. >

Right: When scientists have to improvise ... a simple folding table – and an ironing board – serve Valenzano and his colleagues as the base for genetic and intestinal analyses in the savanna.

Below: Typical habitat of *Nothobranchius furzeri* in Gonarezhou National Park in Zimbabwe during the dry period. Most bodies of water dry up completely during this season. The killifish eggs wait in the desiccated ground for the next rain.





The researchers working with Valenzano have found *Nothobranchius furzeri* in various locations in Mozambique, and recently also in Gonarezhou National Park in Zimbabwe (GRZ). The climates at these various locations differ considerably: a tropical monsoon climate in the lowlands (blue), a desert climate in the highlands (red). The drier the climate, the faster the ponds dry up again, which could explain the particularly short life span of the killifish living in Gonarezhou Park.

Other fish, in contrast, wouldn't dream of sacrificing their longevity. Lungfish, for example, which live in the same ponds as *Nothobranchius*, burrow deep into the mud and wait there 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 found yet another solution to the problem: some North American killifish jump out of drying ponds and survive the dry period on land in damp wood.

OUT OF THE LAB AND INTO THE SAVANNA

"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 the fish as they occur in nature. However, until the turn of the millennium, science labs held only 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 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 (after the park in which they were found, Gonarezhou National Park in Zimbabwe), have bred only with individuals of the same strain during this whole time, the fish have become extremely similar genetically. 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.

Fish of the GRZ strain have the shortest life expectancy of all vertebrates that can be bred in captivity: 9 weeks on average to 13 weeks at most under controlled laboratory conditions.

For a long time, it remained unclear whether the short life span of the GRZ strain was a consequence of decades of inbreeding or whether *Nothobranchius furzeri* has a similarly short life span in the wild.

In 2004, Valenzano and several colleagues thus 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 there: regions at higher altitudes in the interior of the country are relatively dry compared with the coastal lowlands, which receive more rain. The coastal areas thus don't 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 examine whether

» Cancer is the most common cause of death in laboratory killifish.

different natural climatic conditions affect the aging process.

The wild fish live for 25 to 32 weeks, significantly longer than the GRZ fish from the laboratory. However, they still have a very short life expectancy for a vertebrate. What's more, the highland fish do, indeed, age more quickly and die sooner than the fish from the wet 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 east of Zimbabwe, a region that is even drier than the habitats in Mozambique. "So the breeding conditions in the laboratory aren't necessarily what make the GRZ strain so short-lived. Perhaps the extreme shortness of their life span is due to the extreme dryness of their natural habitat," Valenzano says.

CAPTURING FISH IN A NATURE RESERVE

To clarify this point, Valenzano had to journey to Zimbabwe's Gonarezhou National Park, the original home of the GRZ strain. It took five years to gather the necessary papers from the national park authorities, despite the fact that he didn't want 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 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. Before entering the national park, however, they made a quick stop in a hardware store to buy an ironing board. "It wasn't for our laundry, of course. We needed a flat surface on which we could collect our samples. A collapsible ironing board fit the bill perfectly," Valenzano remembers with a smile.

With the samples from Gonarezhou, Valenzano can now compare the genome of the GRZ strain with that of the fish from Mozambique. He hopes to glean further information about which genes control the aging process in *Nothobranchius furzeri*. He also aims to analyze how often the various alleles of aging-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 the wild fish.

To do that, 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 perfectly feasible for a young scientist. His aquariums will then contain fish of the 40th or 60th generation. Translated into human life spans, that corresponds to 1,000 to 1,500 years. Humans clearly can't hold a candle to the killifish as a model organism for aging research. ◀

TO THE POINT

- Thanks to the shortest life span of all vertebrates that can be bred in the laboratory, *Nothobranchius furzeri* is eminently suitable as a model organism for studying aging processes.
- Scientists around the world are studying which genes control the aging process in *Nothobranchius*. To this end, they artificially induce genetic mutations and study gene variants that occur in nature.
- The life span of *Nothobranchius* is also influenced by other factors besides genes, such as temperature and food availability. The composition of the intestinal flora also plays a role.

GLOSSARY

CRISPR/Cas9: A new molecular biological method that enables scientists to genetically modify organisms much more easily and inexpensively than before. The CRISPR/Cas system serves as a sort of immune system in bacteria, neutralizing viruses – so-called bacteriophages. Cas enzymes can cut DNA at repeating segments known as CRISPRs (clustered regularly interspaced short palindromic repeats). Researchers can then insert new DNA segments at these sites.

Killifish: They belong to the taxon of tooth-carps. The name stems from the Dutch word for drainage ditch (*kill*). The fish were discovered in such ditches in the Dutch colonies of North America. Killifish are the egg-laying species of tooth-carps. This classification is now scientifically obsolete, but the term is still commonly used by aquarists.

The Diversity of Aging



HOW HUMANS AGE

Example:
Japanese woman
in 2009

The mortality risk is initially
relatively constant, but rises
steeply with increasing age.

**Increasing
MORTALITY RISK**

Humans are most fertile
in their younger years.

FERTILITY

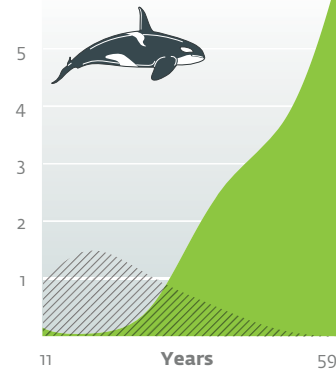
MORTALITY RISK

15 Years 102

TYPICAL LIFE EXPECTANCIES



ORCA



Many animals age
similarly to humans

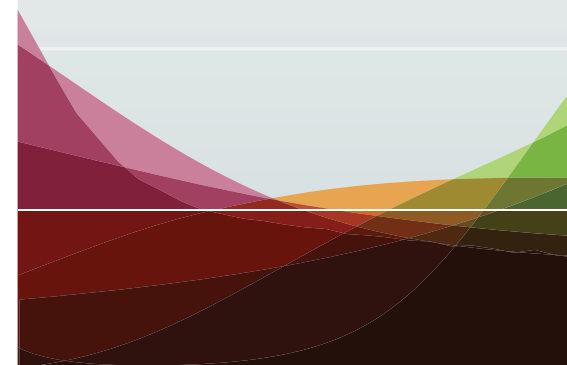
NEMA- TODE



AGING CURVES

Relative mortality risk (colored areas)
and fertility (gray hatchings) in
relation to age, beginning with
sexual maturity up to the age
at which only 5 percent of adult
individuals are still alive.

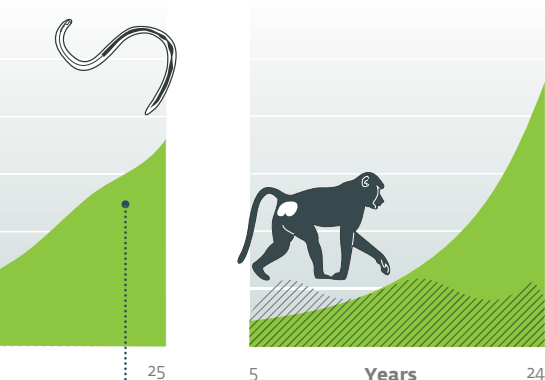
Mortality risk and fertility are shown
in the remaining graphs in relation to
the respective life average.



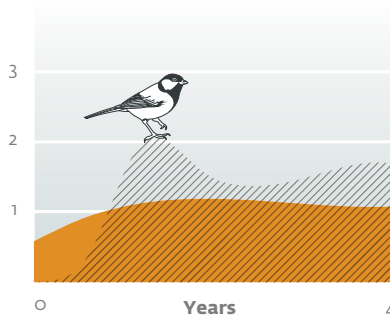
← Age of sexual maturity

**Constant
MORTALITY RISK**

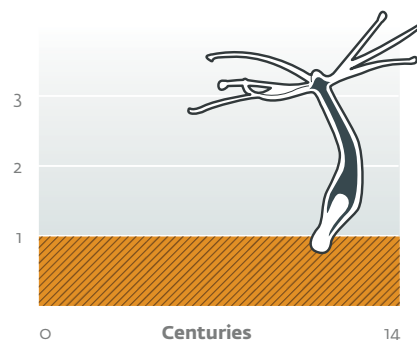
YELLOW BABOON



GREAT TIT



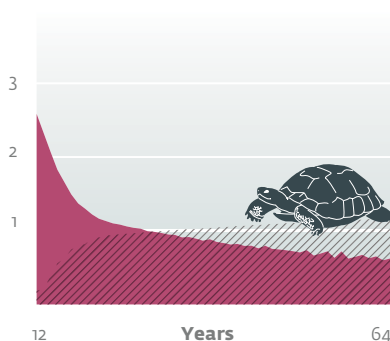
FRESHWATER POLYP



**Decreasing
MORTALITY RISK**

Some plants and animals die less frequently the older they become.

**CALIFORNIA
DESERT TORTOISE**



**OAK
(QUERCUS RUGOSA)**

