Overview

The African Turquoise Killifish: A Scalable Vertebrate Model for Aging and Other Complex Phenotypes

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The African turquoise killifish *Nothobranchius furzeri* is currently the shortest-lived vertebrate that can be bred in captivity. Because of its short life span of only 4–6 months, rapid generation time, high fecundity, and low cost of maintenance, the African turquoise killifish has emerged as an attractive model organism that combines the scalability of invertebrate models with the unique features of vertebrate organisms. A growing community of researchers is using the African turquoise killifish for studies in diverse fields, including aging, organ regeneration, development, "suspended animation," evolution, neuroscience, and disease. A wide range of techniques is now available for killifish research, from genetic manipulations and genomic tools to specialized assays for studying life span, organ biology, response to injury, etc. This protocol collection provides detailed descriptions of the methods that are generally applicable to all killifish laboratories and those that are limited to specific disciplines. Here, we give an overview of the features that render the African turquoise killifish a unique fast-track vertebrate model organism.

FILLING THE MODEL ORGANISM GAP—A SCALABLE, SHORT-LIVED VERTEBRATE MODEL

The quest to understand life has been propelled by the development and use of model organisms. Focusing on specific model species has provided key insights into biological processes while generating a common set of resources and tools that can be shared across laboratories worldwide. Classical nonvertebrate model organisms, such as the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster* have allowed the identification of the genetic pathways involved in cell cycle, cell death, development, and aging. In parallel, studies in vertebrate models such as the mouse *Mus musculus*, the African clawed frog *Xenopus laevis*, and the zebrafish *Danio rerio* have helped to achieve understanding of vertebrate development and diseases.

Despite the strengths of these models, until recently, there was no complex vertebrate model species with the advantages of scalability, rapid development, and short life span that many invertebrate species offer. The lack of such a model has been a major roadblock for aging and disease research and for research questions that require phenotyping tens or even hundreds of mutants (e.g., genomic or drug screens). Over the past two decades, the African turquoise killifish *Nothobranchius furzeri* has been established as a model that fills this important gap (Valdesalici and Cellerino 2003; Genade et al. 2005; Terzibasi et al. 2007; Harel and Brunet 2015; Cellerino et al. 2016; Kim et al. 2016; Cui et al.

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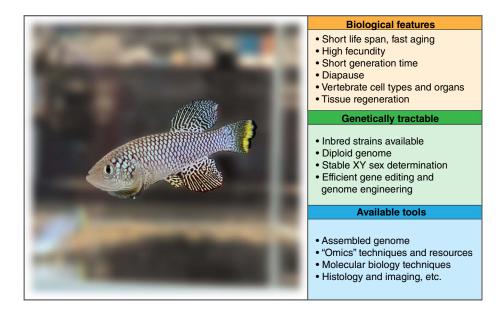| Biological features |
| --- |
| • Short life span, fast aging
• High fecundity
• Short generation time
• Diapause
• Vertebrate cell types and organs
• Tissue regeneration |
| **Genetically tractable** |
| • Inbred strains available
• Diploid genome
• Stable XY sex determination
• Efficient gene editing and
  genome engineering |
| **Available tools** |
| • Assembled genome
• "Omics" techniques and resources
• Molecular biology techniques
• Histology and imaging, etc. |

FIGURE 1. The African turquoise killifish is a fast-track vertebrate model organism that combines a range of biological features and available experimental tools that render it a unique vertebrate model for studying many areas of science, including aging and age-related diseases, organ regeneration, development, "suspended animation," evolution, and neuroscience.

2020). Breeding and maintaining killifish in a laboratory is easy and inexpensive for a vertebrate organism. The African turquoise killifish has a convenient generation time of 2–2.5 months and an exceptionally short life span of 4–6 months. Killifish have an XY sex-determination system, more similar to mammals than many other fish species, including zebrafish. Efficient genetic manipulation techniques and inbred strains are available. In addition, the African turquoise killifish has unique biological traits; for example, during embryonic development, it has a nonobligate diapause state (a form of "suspended animation") that can preserve complex organs for months or even years (Martin and Podrabsky 2017; Hu and Brunet 2018). These features of the African turquoise killifish have been leveraged to gain knowledge in fields as diverse as aging, regeneration, development, evolution, neuroscience, and disease modeling (Fig. 1).

FROM THE NATURAL HABITAT TO THE LABORATORY

The African turquoise killifish *N. furzeri* was first collected and identified as a species in 1969 (Furzer 1969; Jubb 1971). In the wild, the African turquoise killifish live in seasonal ponds in Zimbabwe and Mozambique, East Africa, where a brief rainy season is followed by a longer dry season (Reichard et al. 2009; Kim et al. 2016; Hu and Brunet 2018). The ephemeral ponds form during the rainy season, providing a suitable environment for the killifish to hatch, grow rapidly, and reproduce before the water dries out. During the dry season, the African turquoise killifish embryos enter diapause—a suspended developmental state that allows them to survive in dry mud until the rain returns (Blažek et al. 2013; Furness et al. 2015; Hu et al. 2020). Because of these unique environmental conditions, the African turquoise killifish evolved a relatively short life span in comparison to other laboratory vertebrates. For example, the common inbred "GRZ" laboratory strain, which originated from a collection in 1970 (Parle 1970), has the shortest recorded median life span of 4–6 months among all killifish strains and species (Valdesalici and Cellerino 2003; Genade et al. 2005; Terzibasi et al. 2008; Kirschner et al. 2012; Hu et al. 2020; McKay et al. 2022). The strains derived from more recent collections, such as the MZM0403 and 0410 strains (which originated from a collection in 2004), have slightly longer median life spans ranging from 8 to 12 months (Terzibasi et al. 2008; Kirschner et al.

2012; Valenzano et al. 2015), but even so, such median life spans remain severalfold shorter than those of canonical vertebrate models, including mice (∼2.5 years; Flurkey et al. 2007) and zebrafish (∼3.5 years; Gerhard et al. 2002). Similarly, the *maximum* life span of the African turquoise killifish (1.1 year; Tozzini et al. 2013) is severalfold shorter than that of mice (4 years; Miller et al. 2002) and zebrafish (5.5 years; Gerhard et al. 2002). Importantly, in a laboratory environment, the life span of the African turquoise killifish stays short over generations. The use of annual fish species for aging research was proposed early on (Liu and Walford 1966; Cooper et al. 1983). Since Alessandro Cellerino's group first published the life span of African turquoise killifish in 2003 (Valdesalici and Cellerino 2003), the killifish community has grown to include many laboratories with active killifish research projects worldwide. The short-lived nature of the African turquoise killifish incentivized the rapid establishment of this species into a laboratory research model for vertebrate aging.

USING THE AFRICAN TURQUOISE KILLIFISH FOR DISCOVERIES ACROSS BIOLOGY

Aging and Life Span

Unique Advantages of the African Turquoise Killifish Aging Model

The compressed aging process of the African turquoise killifish makes this organism an exciting model with which to study the biology of aging (Valdesalici and Cellerino 2003; Kim et al. 2016). The killifish has vertebrate-specific cell types, organs, and systems that play crucial roles in the aging process but that are absent from invertebrate models such as *C. elegans* and *Drosophila*—for example, an adaptive immune system, a closed circulation system with a functional heart, and a segmented brain with a hypothalamic-pituitary axis (Shimeld and Holland 2000; Flajnik and Kasahara 2010; Stephenson et al. 2017; Singh et al. 2019).

There are clear benefits of studying aging using the African turquoise killifish over canonical vertebrate models (e.g., mice, zebrafish). First, it is feasible to perform longitudinal studies over an animal's entire life. For example, longitudinal transcriptomic analyses on the same individual can be performed by drawing blood or clipping off a piece of the tail when the fish is young and again in old age. Such analyses revealed that the transcriptome profile at a young age is predictive of life span (Baumgart et al. 2016; Kelmer Sacramento et al. 2020). Second, one can rapidly assess how experimental interventions affect life span. Recently, the African turquoise killifish has been used for high-throughput studies and to test how diet regimens, drugs, or the gut microbiome affect life span (Valenzano et al. 2006; Smith et al. 2017; McKay et al. 2022). These studies illustrate the promise of the killifish model to identify the molecular players that regulate vertebrate aging and to experimentally test whether modulating these players can reduce aging phenotypes and mortality. Lastly, genetic mutants can be easily generated, opening the prospect of using the killifish to study human disease variants, especially in the context of age-related diseases including many neurodegenerative diseases.

Aging Phenotypes

Old killifish show phenotypes that recapitulate many of the classical hallmarks of aging, such as kyphosis (curvature of the spine), color loss, sarcopenia (loss of muscle strength), reduced locomotion, neurodegeneration and signs of cognitive impairment, decreased fecundity, and reduced regenerative capacity (Genade et al. 2005; Valenzano et al. 2006, 2009; Di Cicco et al. 2011; Valenzano et al. 2015; Api et al. 2018; Žák and Reichard 2021). At the molecular level, aging features that are well-known in mammals also occur in killifish, including telomere shortening, mitochondrial dysfunction, and progressive loss of protein homeostasis (Hartmann et al. 2009, 2011; Baumgart et al. 2016; Kelmer Sacramento et al. 2020; Reichard et al. 2022b).

There is also intrapopulation heterogeneity for aging. For example, the African turquoise killifish shows sexual dimorphism in aging phenotypes. Age-dependent reproductive decline is

more pronounced in females than in males (Api et al. 2018; Žák and Reichard 2021). Males and females can also have different life spans, depending on social and environmental factors. Group-housed females have longer median life span (and longer telomeres) than group-housed males (Reichard et al. 2022a,b). However, when the female fish are individually housed, results depend on animal facilities and diet: Females either have a similar life span to males (Valenzano et al. 2015), a longer life span (Teefy et al. 2023), or a shorter life span in an environment of strong dietary restriction (McKay et al. 2022). The set of factors and parameters that contributes to the manifestation of sex-specific aging and life span of the African turquoise killifish is not completely clear yet.

Neurodegeneration and Brain Aging

Neurodegenerative diseases such as Alzheimer's and Parkinson's disease are among the most prevalent diseases in older populations. Hence, age-related changes in the brain have received particular attention in the aging field (Wyss-Coray 2016). Old turquoise killifish show cognitive decline, and several assays have been developed to examine cognitive function in killifish (Valenzano et al. 2006; McKay et al. 2022). Age-related changes in protein abundance and aggregation have also been systematically quantified in the turquoise killifish brain, revealing that many proteins have the propensity to form aggregates in old brains (Kelmer Sacramento et al. 2020; Chen et al. 2022; Harel et al. 2022). Old turquoise killifish also appear to show α-synuclein-containing inclusion bodies, a pathological marker for Parkinson's disease and other diseases in humans (Matsui et al. 2019). Thus, the killifish could be a useful model to identify not only pathological mechanisms but also interventions to counter neurodegenerative diseases.

Suspended Animation

Intriguingly, although the African turquoise killifish has been mostly used in the aging field because of its rapid generation time and fast aging, its development includes a stage of seemingly opposite characteristics: African turquoise killifish embryos can enter diapause, a state of "suspended animation" that enables them to endure the extreme environment of a dried-out pond for several months. Diapause is nonobligatory and can be skipped. Importantly, diapause can occur at three different stages of embryonic development: Diapause I occurs in an early dispersed cell phase, diapause II occurs after the embryonic axis and several complex organs have already formed, and diapause III occurs in fully developed embryos that are poised to hatch (Martin and Podrabsky 2017; Dolfi et al. 2019; Hu et al. 2020; Abitua et al. 2021). Diapause II is the form of diapause that can last the longest.

The transition between active development and diapause in *Nothobranchius* species is dependent on environmental conditions, most notably lack of oxygen for diapause I (Peters 1963; Inglima et al. 1981; Levels et al. 1986; Naumann and Englert 2018; Polačik et al. 2021) and temperature for diapause II (Furness et al. 2015). Some of the pathways that control diapause II entry in response to temperature have been identified in the South American killifish *Austrofundulus limnaeus* (Romney et al. 2018). Comparative studies between killifish species that undergo diapause II and those that do not have provided insights into the evolution of diapause in killifish (Wagner et al. 2018; Singh et al. 2021).

When African turquoise killifish embryos enter diapause II, they undergo a rewiring of gene regulatory programs, which allows for long-term preservation of complex organs through metabolic and epigenomic adaptations (Hu et al. 2020; Singh et al. 2021). Embryos can stay in diapause II for several months or even years (longer than the adult life span), without negatively impacting adult growth, fertility, or life span (Hu et al. 2020). Thus, diapause not only promotes survival under extreme conditions, but it also suspends the aging process. Studying the underlying mechanisms of diapause could provide new insights that might be leveraged to stop the aging clock for long-term organismal preservation.

Regeneration

Teleost fishes can efficiently regenerate many different organs and tissues that have been lost because of injury or disease. In contrast, mammals (including humans) have limited regenerative capacity, even though their genomes contain nearly all genes that have been implicated in the fish regeneration process. The African turquoise killifish can regenerate organs or tissues—for example, upon tail fin amputations, heart injuries (Wendler et al. 2015), or optic nerve or brain injury (Van houcke et al. 2021; Vanhunsel et al. 2021). Comparing the African turquoise killifish's regenerative response to those of other regeneration-competent (e.g., zebrafish) or less competent (e.g., mice) species has identified some of the conserved molecular mechanisms that regulate regeneration-responsive gene expression (Wang et al. 2020). Interestingly, regenerative capacity declines with age in killifish and becomes more "mammalian-like" (Vanhunsel et al. 2022a,b). Deeper understanding of the molecular networks that govern regeneration in the African turquoise killifish will not only shed light on the evolution of regenerative traits, but also guide the development of therapeutic strategies for organ repair in mammals, including wound healing or recovery from heart attack, stroke, or traumatic brain injury.

Evolution

Natural populations of different killifish species live in different habitats across the world. Hence, there is substantial genetic variability among different killifish species and among wild populations of the same species. Life span and diapause duration also differ considerably among species. For example, species that live in harsh habitats (e.g., the African turquoise killifish *N. furzeri* and the South American killifish *A. limnaeus*) have diapause and a short life span in the wild, whereas species that live in a habitat with water throughout the year (e.g., another African killifish *Aphyosemion striatum*) do not have diapause and have a long life span (Wagner et al. 2018; Cui et al. 2019; Singh et al. 2021). Life span can differ even between different strains of the shortest-lived killifish representative *N. furzeri* (Terzibasi et al. 2008; Tozzini et al. 2013; Valenzano et al. 2015; Blažek et al. 2017). This "natural experimental setting" offers a system with which to address fundamental evolutionary processes, as well as to identify genes or regulatory regions that are under positive or purifying selection for their contribution to life span and aging. For example, comparative genomic studies of different long- and short-lived killifish species (Cui et al. 2019) or strains of the same species (Valenzano et al. 2015; Willemsen et al. 2020) have provided evidence that the primary driving force behind the evolution of short life span may be the relaxation of purifying selection against deleterious gene variants in annual species, combined with population bottlenecks that exacerbated mutation accumulation.

AVAILABLE RESOURCES AND TECHNIQUES

Genome

Genome Features

The genome of the African turquoise killifish is estimated to be ∼1.53 Gb, encompassing 22,236 annotated protein-coding genes on 19 synteny groups ("chromosomes") (Cui et al. 2020; Willemsen et al. 2020). Because of the vertebrate and the teleost genome duplication, many African turquoise killifish genes have extra paralogous genes (Reichwald et al. 2015; Singh et al. 2021). Repetitive regions comprise up to ∼66% of the genome, with high prevalence of transposable elements (Reichwald et al. 2015; Valenzano et al. 2015; Willemsen et al. 2020).

In the African turquoise killifish (like in mammals), females are the homogametic sex (XX) and males are the heterogametic sex (XY) (Valenzano et al. 2009; Platzer and Englert 2016). The sex-determining region is located on synteny group 05 (Sgr05). In this region, females have two identical copies of the TGF-ß family gene *gdf6* (*gdf6/gdf6*), a putative sex-determining gene, whereas males have one copy of *gdf6* and an altered copy of *gdf6* named *gdf6Y* (*gdf6/gdf6Y*) (Reichwald et al. 2015).

The mitochondrial genome of the African turquoise killifish contains the same genes as the mitochondrial genomes of most animals, with one additional copy of a transfer RNA gene.

It encodes 13 proteins (all subunits of respiratory chain complexes), two ribosomal RNAs, and 23 transfer RNAs in the vertebrate-typical gene arrangement (Boore 1999; Hartmann et al. 2011).

Genome Resources

The reference genome and annotated genome files (e.g., GTF file) of the African turquoise killifish are available on the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/genome/2642/). The most recent reference genome was released in 2020 (Willemsen et al. 2020) and was built by combining two earlier reference genomes that were de novo assembled by Anne Brunet's laboratory in Stanford, USA (Valenzano et al. 2015) and Matthias Platzer's laboratory in Jena, Germany (Reichwald et al. 2015). The newest version of the genome has higher gene coverage and improved continuity compared to the earlier assemblies. The Genome Data Viewer from NCBI (https://www.ncbi.nlm.nih.gov/) allows users to select a specific reference genome assembly to display, either the 2020 version (MPIA_NFZ_2.0) or the 2016 version (Nfu_20140520). As the transcriptomic, proteomic, and epigenomic data sets published before 2020 were mapped to the 2016 version, the flexibility of interchanging the genome versions on the Data Viewer allows users to benefit from a more comprehensive genome coverage while integrating data from prior studies.

In addition to a reference genome, other genetic resources have also been established, including quantitative trait loci, genetic linkage maps, and microsatellite markers (Valenzano et al. 2009; Kirschner et al. 2012; Bartáková et al. 2013; Valenzano et al. 2015).

An increasing number of transcriptomics, epigenomics, and proteomics data sets both in adult tissues and in embryos have been published. The genomic data sets are generally available on the Gene Expression Omnibus (GEO), an NCBI data repository (https://www.ncbi.nlm.nih.gov/geo/). A search using key words, such as "*N furzeri* RNA-seq," on the NCBI website returns the currently available data sets. Most publications also have the "GEO accession number" (e.g., GSE150318) associated with the published data set. Searching using the GEO accession number allows access to a given published data set. The proteomics data sets are generally available on ProteomeXchange and can be accessed by using 'Nothobranchius furzeri' as a keyword (http://www.proteomexchange.org/).

Genetic Manipulation

Efficient engineering of the African turquoise killifish genome has been developed, including the generation of knockout and loss-of-function mutants using the CRISPR–Cas9 system (Harel et al. 2015, 2016), as well as the introduction of transgenes into the African turquoise killifish genome using Tol2-mediated transposons (Valenzano et al. 2011; Hartmann and Englert 2012; Allard et al. 2013) or CRISPR–Cas9-mediated gene knock-in (Nath et al. 2022; Oginuma et al. 2022; Krug et al. 2023). A CRISPR–Cas13 system has been established for knockdown of mRNA (Kushawah et al. 2020). These tools, combined with the short generation time of the killifish, allow high-throughput genetic studies, with generation of multiple stable mutant lines.

The killifish field is also actively developing additional genetic manipulation strategies, such as (1) conditional gene repression and activation tools that act in cell-type- and/or temporal-specific manners, and (2) precise alteration at the base pair level to introduce disease variants. Such technological advancements will open exciting new fronts to study vertebrate biology and model human diseases using the African turquoise killifish. This model offers a highly scalable vertebrate platform that uniquely complements the features of classical vertebrate model organisms.

OVERVIEW OF THE PROTOCOL COLLECTION

This entire article collection covers seven major areas, including general protocols that most killifish laboratories would use and specialized protocols for specific research topics. The general protocols are organized into three sections: (1) Husbandry, Reproduction, and Preservation, which presents

methods to establish, maintain, and preserve a colony of African turquoise killifish; (2) Genetic Manipulation and Analysis, which describes genome-editing tools such as the CRISPR–Cas9 system, Tol2-mediated transgenesis, and rapid genotyping methods for embryos and juvenile fish; and (3) Genomics, which details methods for genomics techniques such as RNA sequencing, transposon mapping, microbiota profiling, and specialized gene locus sequencing.

The next four sections include protocols that are specific to a given research area. These sections include (4) Aging and Life-Span Analysis, which describes methods to measure killifish life span and perform molecular, biochemical, and morphological analyses of aging hallmarks; (5) Tissue Analysis (General, Fat, Blood, Brain), which covers protocols to perform histology in multiple tissues, fat analysis, blood sampling, and immunofluorescence assays and single-cell dissociation for the brain; (6) Nervous System Injury Methods, which describes protocols to study tissue regeneration and repair in the brain; and (7) Embryo Development and Diapause, which covers techniques specific to studying embryos.

NOMENCLATURE

In this protocol collection, we will use several terms interchangeably: *Nothobranchius furzeri*, *N. furzeri*, African turquoise killifish, turquoise killifish, and sometimes only killifish for simplicity. However, we note that the terms "killifish" or "African killifish" can be ambiguous because there are other killifish or African killifish species that have different characteristics compared to the African turquoise killifish (e.g., in terms of longevity and presence of diapause II). We also note that other killifish species (e.g., the South American killifish *A. limnaeus*) are used for diapause studies (and some of the protocols described here may be relevant for other killifish species).

In this protocol collection, we will use the term "fry," "juvenile," and "larvae" interchangeably to denote recently hatched killifish. We note that killifish, unlike zebrafish, do not really have a larval stage and hatch directly as juvenile fish.

Overall, this protocol collection should be a valuable resource not only for newcomers who wish to start African turquoise killifish colonies in their laboratories, but also for established killifish laboratories that want to optimize their experimental toolkit for research on a unique vertebrate model.

ACKNOWLEDGMENTS

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