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From the bush to the bench: the annual *Nothobranchius* fishes as a new model system in biology

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ABSTRACT

African annual fishes from the genus *Nothobranchius* are small teleosts that inhabit temporary water bodies subject to annual desiccation due to the alternation of the monsoon seasons. Given their unique biology, these fish have emerged as a model taxon in several biological disciplines. Their increasing popularity stems from the extremely short lifespan that is the result of their specific life-history adaptations and is retained under laboratory conditions. *Nothobranchius furzeri*, the most popular laboratory species, is the vertebrate species with the shortest lifespan recorded in captivity. In the laboratory, adults of different *Nothobranchius* species and populations live between 3 and 18 months and, notably, there is a negative correlation between the captive lifespan of a species and the aridity of their habitat. Their short lifespan is coupled to rapid age-dependent functional decline and expression of cellular and molecular changes comparable to those observed in other vertebrates, including humans. The recent development of transgenesis in this species makes it possible to insert specific constructs into their genome, and the establishment of transgenic lines is facilitated by their very rapid generation time, which can be as short as 1 month. This makes *Nothobranchius* species particularly suited for investigating biological and molecular aspects of ageing and ageing-associated dysfunctions. At the same time, they also represent a unique model taxon to investigate the evolution of life-history adaptations and their genetic architecture. We review their natural history, including phylogenetic relationships, distribution in relation to habitat conditions and natural selection for differential longevity, population structure and demography, and life cycle with emphasis on diapause that may occur at three stages during embryonic development. We further critically evaluate their use as a laboratory model for understanding the evolution of a rapid ageing rate and its consequences for other life-history traits, for cellular, molecular and integrative traits associated with the ageing process, high incidence of neoplasias, their utility for genome-wide gene-expression studies, and as a model for quantitative genetics. We summarize recent achievements in fostering *Nothobranchius* species as a widely applicable model system, including an annotated transcriptome, successful transgenesis, and existence of viable inbred lines. We compare the conditions they experience in the wild and in captivity and suggest that they are an ideal taxon to investigate natural genetic variation in a laboratory setting. We conclude that *Nothobranchius* species – and *N. furzeri* in particular – could become a unique model taxon that bridges interests in ecological and biomedical research. We hope that a conceptual and methodological integration of these two branches of biology will provide important new insights.

Key words: ageing, longevity, killifish, annual fish, diapause, inbred lines, life-history traits, quantitative genetics, model species, senescence.

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I. INTRODUCTION

Annual killifish are a group of teleost fishes from the cyprinodont clade (order Cyprinodontiformes), a diverse taxon with primarily Gondwanan distribution. Cyprinodontiforms are small fish and regularly occupy marginal habitats that are often inhospitable for other teleost fishes (Naiman, Gerking & Ratcliff, 1973; Taylor *et al.*, 2008). Annual killifish are adapted to a unique environment of temporary savannah pools in Africa and in the Neotropics. Their populations survive in a habitat that desiccates annually during the dry season and is filled by rainwater during the wet season.

Most of the African annual killifishes belong to the genus *Nothobranchius* (Nothobranchiidae), which comprises over 60 described species distributed throughout East and Central Africa (Froese & Pauly, 2014). Closely related species-poor genera of annual fish, *Pronothobranchius* and *Fundulosoma* from the Sahel region of West Africa (Valdesalici, 2013), their sister taxa, are sometimes included in the genus *Nothobranchius* (Wildekamp, 2004). African annual killifishes are small (3–15 cm total length, typically <7 cm), with marked sexual dimorphism and dichromatism. They have a short maximum natural lifespan (<12 months) due to annual desiccation of the pools in which they live. Notably, this short lifespan is also retained in captivity, varying between 3 and 18 months (Valdesalici & Cellerino, 2003; Lucas-Sánchez *et al.*, 2011; Baumgart *et al.*, 2015). The *Nothobranchius* life cycle is entirely adapted to the ephemeral and unpredictable conditions of their habitat. Fish hatch when the pool is filled with water, grow rapidly and become sexually mature within a few weeks (Polačik, Donner & Reichard, 2011; Blažek, Polačik & Reichard, 2013). After reaching sexual maturity, they reproduce daily. Eggs are spawned into the muddy substrate of the pool and remain there after the pool desiccates. Embryos survive throughout the dry period in

developmental diapause and their hatching is triggered by extrinsic factors when the habitat is flooded again.

The same ecological guild of killifish occurs also in the Neotropics; in the dry region including Gran Savanna (Colombia, Venezuela) and Cerrado (Brazil) north of the equator (Nico & Thomerson, 1989) and in the temperate Pampas ecosystem south of the equator (Berois, Arezo & de Sá, 2014). Neotropical annual killifishes share several adaptations to seasonal pools with African *Nothobranchius* species (e.g. facultative diapauses at corresponding developmental stages), but also possess unique features (e.g. in their reproductive behaviour). The life history of annual killifishes makes them an ideal group to address research questions in several areas of biological sciences.

II. *NOTHOBRANCHIUS*: A HISTORICAL PERSPECTIVE

The first published scientific research on *Nothobranchius* annual fish dates to the 1970s and 1980s. *Nothobranchius guentheri* (Pfeffer) were first studied in the context of sexual selection by Richard Haas, who investigated the role of the bright colouration in males in sexual and natural selection (Haas, 1976*b*). *N. guentheri* were then used in a series of explorative ageing studies by Mathias and Markofsky, who first described the age-dependent onset of histopathological lesions in several organs of *Nothobranchius* (Markofsky & Perlmutter, 1972; Markofsky, 1976; Markofsky & Matias, 1977; Markofsky & Milstoc, 1979*a*,*b*). At the same time, Walford and Liu studied the South American annual species *Austrolebias bellottii* (Steindachner) describing their lifespan and histopathological lesions (Liu & Walford, 1969). Interestingly, age-associated changes in *A. bellotti* were very similar to those observed in *N. guentheri*. These authors also described the effects of water temperature on growth and ageing in this species (Liu & Walford, 1970, 1972, 1975; Liu, Leung & Walford, 1975). However, annual killifish never became fully established as model organisms, possibly because of the relatively long lifespan (exceeding 1 year) of the species then available (as compared to *N. furzeri*) and the minor role played by fish models in biomedical research at that time. In 1972, a series of three papers (Wourms, 1972*a*,*b*,*c*) described the peculiar embryogenesis and diapause of annual and non-annual genera of cyprinodonts. Further studies investigated specifically cleavage and early cell movements in *Nothobranchius* spp. as well as the effects of some environmental variables on diapause (Van Haarlem, Van Wijk & Fikkert, 1981; Van Haarlem, Konings & Van Wijk, 1983a; Van Haarlem, Van Wijk & Konings, 1983b; Levels, Gubbels & Denuce, 1986). *Nothobranchius rachovii* Ahl was also proposed as a model for genotoxicology due to its small number of chromosomes (2n = 16) (van der Hoeven *et al.*, 1982). Interest in *Nothobranchius* species revived after 2003 due to the description of the extremely short lifespan of the GRZ strain of *Nothobranchius furzeri* Jubb in captivity (Valdesalici & Cellerino, 2003).

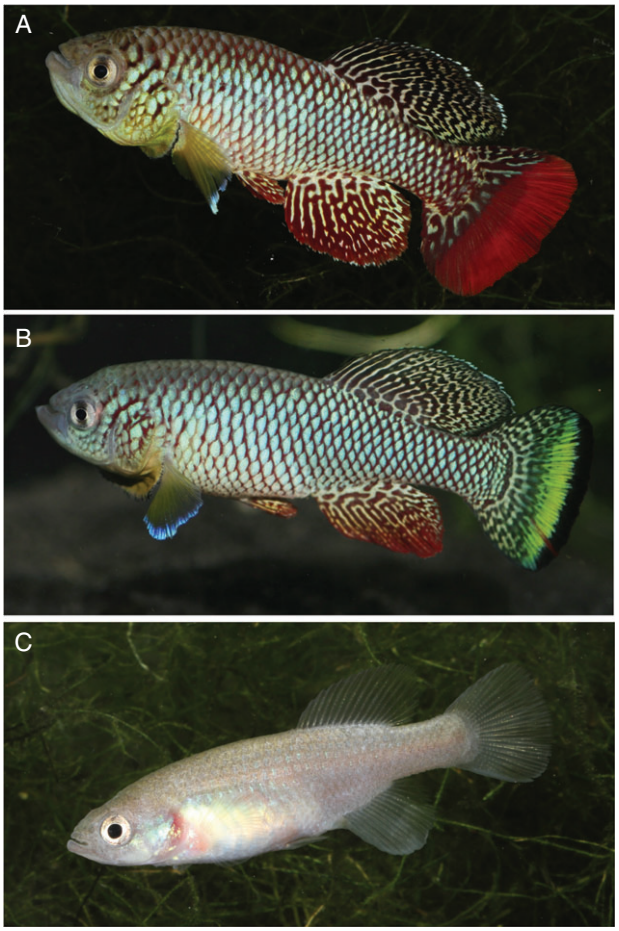

Fig. 1. Adult males of (A) red and (B) yellow morphs, and (C) female *Nothobranchius furzeri*.

Since most genetic research on *Nothobranchius* is conducted in *N. furzeri*, the present review will cover mainly this species (Fig. 1). However, other species of annual fish will also be discussed when appropriate.

III. DISTRIBUTION, ECOLOGY AND POPULATION STRUCTURE

(1) Phylogeny, distribution and habitat of the genus

The distribution of the genus *Nothobranchius* ranges from Sudan in the north to KwaZulu Natal in South Africa, and includes the eastern part of the continent (Wildekamp, 2004). *Nothobranchius* is composed of four well-defined phylogenetic clades that are almost exclusively allopatric (Dorn *et al.*, 2014). The basal northern clade inhabits particularly dry regions in northern Kenya and Somalia. The southern clade is distributed south of the Zambezi River, across a gradient from a humid coastal zone to dry habitats at higher altitudes. This clade is the most researched, with expanding knowledge of phylogenetic relationships (Terzibasi *et al.*, 2008; Shidlovskiy, Watters & Wildekamp,

2010), phylogeography and population genetics (Dorn *et al.*, 2011; Bartáková *et al.*, 2013) as well as ecology (e.g. Reichard, Polačik & Sedláček, 2009; Polačik & Reichard, 2010; Reichard *et al.*, 2014). The inland clade is distributed in a high-altitude region between Lake Victoria in Uganda and Kafue basin in Zambia and separated from the coastal clade (coastal basins in southern Kenya, Tanzania, and northern Mozambique) by rift valleys (Dorn *et al.*, 2014). Separation of the four clades occurred by vicariance events. Secondary calibration (i.e. using information from molecular dating studies on related taxa) revealed that the age of the genus *Nothobranchius* is 8.3 (6.0–10.7) million years (My) and the separation of the four main clades was completed 4.8 (2.7–7.0) million years ago (Mya). Diversification within the clades started approximately 3 Mya and most species pairs appear to have an age of 0.5–1 Mya. Therefore, the origin and early diversification of *Nothobranchius* seems to coincide with the aridification of East Africa and expansion of the savannah biome (deMenocal, 2004; Trauth *et al.*, 2005), similarly to the origin of our own species from pre-human hominins. Climate variation during the late Pleistocene resulted in intra-specific diversification rather than speciation (Dorn *et al.*, 2014). Previous primary calibration using geological events assumed much older divergence times, congruent with major geological uplifting of the African continent and East African rifting (Dorn *et al.*, 2011; Bartáková *et al.*, 2013). The highest species diversity is documented in coastal areas of Tanzania (Wildekamp, 2004).

Despite its seemingly extensive range, *Nothobranchius* distribution is mosaic due to their strict requirements of a suitable soil substrate ensuring egg survival during the dry period (Reichard *et al.*, 2010). *Nothobranchius* distribution is tightly coupled with the presence of Quaternary vertisol soils (Watters, 2009), apparent as a dark mud substrate forming deep cracks when desiccated. *Nothobranchius* pools are found in woodland savannah where topography enables development of small depressions that accumulate water or in flat coastal plain. The woodland savannah pools are typically fed by rain water. Temporary streams or (semi-) permanent rivers are sometimes connected to some of the pools. *Nothobranchius* species are also found in remnant pools within the channels of temporary streams (Reichard *et al.*, 2009; Nagy, 2014). However, it is unclear whether these populations are stable or whether annual killifish in these pools consist of individuals drained from nearby savannah pools. In wetter portions of their range, they occur in inland marshes when pools are part of large grassland savannah matrices (Ng'oma *et al.*, 2013). These pools are less fragmented and may support other fish than *Nothobranchius* species (Watters, 2009). Nowadays, these pools are often converted into rice fields (Ng'oma *et al.*, 2013). In isolated pools, *Nothobranchius* may coexist with lungfish (*Protopterus* spp.). The pools with at least a temporary connection to permanent waters may be colonized by juveniles of *Tilapia sensu lato*, small '*Barbus*' barbs or *Clarias gariepinus* (Burchell) (Reichard, 2010; Nagy, 2014) but never support their stable populations. In the coastal plain, *Nothobranchius* may co-occur with a highly diverse fish community (M. Reichard, unpublished data). Several *Nothobranchius* species may inhabit the same pool (Reichard *et al.*, 2009); up to four species may coexist in the same habitat (Wildekamp, 2004). Sister species are typically allopatric, at least in the well-researched southern clade, but closely related species from the same crown clade often co-occur (Reichard *et al.*, 2009).

(2) Distribution of *Nothobranchius furzeri* and specific habitat conditions

The range of *N. furzeri* is particularly well studied (Fig. 2). It encompasses the area between the Save river in the north and the Lebombo ridge (South Africa) in the south. The type locality is the Sazale Pan (Gonarezhou National Park), Zimbabwe, where the species was first collected in 1968 (Jubb, 1971). The type locality is located 25 km from the Mozambican border. All recent populations of *N. furzeri* have been collected in Mozambique (Reichard *et al.*, 2009, 2014), including one population at the border with Zimbabwe. All imported populations possess unique collection codes. Populations imported from several collection expeditions are available for laboratory work. The GRZ strain originates from the first collection of the species at its type locality in 1968 (Jubb, 1971). All other populations were collected in Mozambique between 1998 and 2012. The list of wild populations currently available as captive strains is presented in Table 1.

The range of *N. furzeri* encompasses a strong cline of aridity and rainfall unpredictability (200–600 mm/year), with different populations experiencing between 2 and 7 months of habitat existence (Terzibasi Tozzini *et al.*, 2013; Polačik *et al.*, 2014*a*). The most arid conditions are found inland (higher altitude, furthest from the ocean); the region receives erratic precipitation and is subject to stronger evaporation (Mazuze, 2007; Terzibasi *et al.*, 2008). Lowland areas closer to the Indian Ocean enjoy a relatively humid climate, with longer and more predictable rainfall patterns (Terzibasi Tozzini *et al.*, 2013). Importantly, populations are deeply structured and the two major lineages are separated in the humid and dry parts of the range.

The range of *N. furzeri* lies within a dry habitat extreme in the range of the genus *Nothobranchius* and consists of small isolated pools separated from active stream channels and alluvial plains (Reichard *et al.*, 2009). Water is typically turbid with high disturbance and organic input from large herbivores. While domestic cattle are currently the only large mammals visiting *N. furzeri*'s habitats in Mozambique, it is likely that the pools served as drinking reservoirs for typical African megafauna in the past. Water may be less turbid and even transparent when vegetation is abundant. *N. furzeri* populations are distributed along an altitudinal gradient that ranges between 16 and 422 m above sea level (a.s.l.), but most populations inhabit areas between 16 and 200 m a.s.l. (Reichard *et al.*, 2009). Only two populations are known from higher altitudes; MZCS 323 (222 m) and GRZ (422 m). Water conductivity (describing total dissolved solids, i.e. water hardness related to its mineral content) in *N. furzeri* habitats varies widely, from 50 to 625 μS/cm, and the substrate is typically very soft mud. Vegetation is often

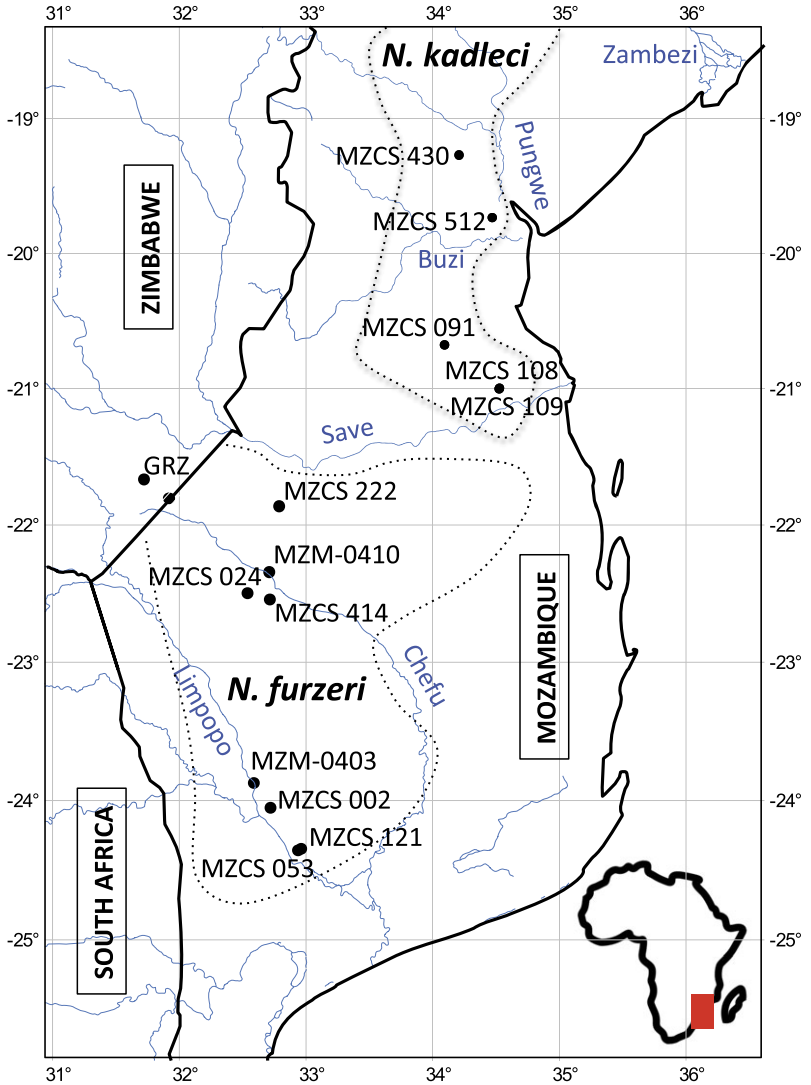

Fig. 2. Distribution of *N. furzeri* and its sister species, *N. kadleci*. Locations of source pools of captive strains are indicated. The range of each species is illustrated by the broken line; note that the extent of the range of *N. furzeri* in Zimbabwe is not known. The position of this study area at the continental scale is indicated in the inset.

present in the form of *Nymphaea* sp., grass vegetation and, occasionally, submergent vegetation (Reichard *et al.*, 2009). Many pools, however, lack any vegetation (Reichard *et al.*, 2014). Anuran larvae often coexist with *N. furzeri* and other *Nothobranchius* species and may dominate over fish in terms of numerical abundance and biomass.

(3) Diet

Nothobranchius, including *N. furzeri*, primarily feed on macroinvertebrates. Taxonomically, their diet appears opportunistic and largely depends on prey availability (Polačik & Reichard, 2010). It contains both pelagic and benthic preys. There is an apparent avoidance of hard-bodied heteropterans (Polačik & Reichard, 2010), though they are consumed when food is scarce. A study on food selectivity in four wild populations of *N. furzeri* indicated that they preferred to feed on small crustaceans (Cladocera, Copepoda, Ostracoda and Conchostraca), while sympatric *N. orthonotus* (Peters) tended to prey on aquatic insect larvae (e.g. Odonata, Ephemeroptera) (Polačik & Reichard, 2010). When several *Nothobranchius* species co-occur, their diets tend to be segregated when resources are not limiting (Polačik & Reichard, 2010; Polačik *et al.*, 2014*b*). Diet composition, however, largely overlaps. All *Nothobranchius* tend to be prey generalists in a broad sense. *Nothobranchius microlepis* (Vicinguerra) and *Nothobranchius jubbi* Wildekamp & Berkenkamp have a preference for small crustaceans and their *nauplii* and frequently consume mosquito larvae and hemipteran nymphs (Wildekamp, 1983; Wildekamp & Haas, 1992). Captive *N. furzeri*, as well as other species of the genus, readily consume live and frozen dipteran larvae such as larvae of the genera *Chironomus* and *Chaoborus* (Genade, 2005). Live *Tubifex* sp. (Oligochaeta), *Artemia salina* and *Daphnia* spp. are readily taken by captive

Table 1. List of captive populations of *N. furzeri* and *N. kadleci* imported from the wild and available in captivity

Strain	GPS coordinates	Collection year	Genetic clade[a]	Founders[b]	Labs[c]	Colour morphs
N. furzeri						
GRZ	21° 40.600′S 31° 44.000′E	1969	Chefu	Unknown	MPI	Exclusively yellow
MZM-0403	23° 53.112′S 32° 36.006′E	2004	Limpopo north	Unknown	FLI, MPI	Exclusively red
MZM-0410	22° 21.290′S 32° 43.305′E	2004	Chefu	Unknown	FLI, SNS	Mainly yellow, red rare
MZM-0703	23° 48.500′S 32° 37.700′E	2007	Limpopo north	Unknown	FLI	Exclusively red
MZCS 222	21° 52.414′S 32° 48.039′E	2011	Chefu	20 + 40	IVB	Red + yellow
MZCS 121	24° 21.471′S 32° 58.446′E	2011	Limpopo north	15 + 28	IVB	Mainly red, yellow rare
MZCS 414	22° 33.278′S 32° 43.635′E	2012	Chefu	7 + 12	IVB	Red + yellow
MZCS 053	24° 22.159′S 32° 57.002′E	2008	Limpopo north	10 + 10	IVB	Mainly red, yellow rare
MZCS 024	22° 30.498′S 32° 33.046′E	2008	Chefu	29 + 16	IVB	Red + yellow
MZCS 002	24° 03.808′S 32° 43.932′E	2012	Limpopo north	7 + 10	IVB	Exclusively red
N. kadleci						
MZCS 108	21° 00.728′S 34° 32.219′E	2008	Save	2 + 2	IVB	NA
MZCS 091	20° 41.277′S 34° 06.365′E	2008	Gorongosa	2 + 3	IVB	NA
MZCS 091	20° 41.277′S 34° 06.365′E	2010	Gorongosa	5 + 5	IVB	NA
MZCS 109	21° 00.439′S 34° 32.366′E	2010	Save	5 + 5	IVB	NA
MZCS 430	19° 16.838′S 34° 13.230′E	2011	Nhamatanda	10 + 20	IVB	NA

[a]Inclusion in a phylogeographic clade *sensu* Bartáková *et al.* (2013).
[b]Number of males + female in founding populations.
[c]FLI, Fritz Lipmann Institute for Age Research, Jena; IVB, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno; MPI, Max Planck Institute of Biology of Ageing, Cologne; SNS, Scuola Normale Superiore, Pisa.

Nothobranchius spp. Cannibalism has been observed in captivity in juvenile fish, especially in *N. orthonotus* younger than 14 days, when individual differences in body size were large. There is no record of cannibalism in the wild (Polačik & Reichard, 2010), but it is expected in juvenile fish whenever size differences exceed approximately 30% or larger.

(4) Sexual dimorphism and male colour polymorphism

All *Nothobranchius* are highly sexually dimorphic and dichromatic; males are robust and colourful while females are dull. The bright male colouration is sexually selected and species-specific (Haas, 1976*b*; Wildekamp, 2004). Males of several species, including *N. furzeri*, occur in two or more colour forms that may be sympatric or allopatric. In *N. furzeri*, red and yellow morphs are present, differing primarily in the colouration of the caudal fin (Fig. 1). The yellow morph always has also a distal black band, whereas the red morph occurs in two forms: one with a distal black band, and the other with a homogeneously red tail. Yellow males are distributed in the northern and southwestern part of the species range, coinciding with two genetic clades. Yellow males typically co-occur with red males. The red male morph is dominant in central part of the species range, largely associated with one of the clades. In the centre of the *N. furzeri* distribution, some populations appear to be composed exclusively of the red morph (Reichard *et al.*, 2009). Males of both forms are typically found in most populations when sampling effort increases (Reichard *et al.*, 2009), yet some populations appear to be exclusively red: even when in excess of 100 males were collected, no yellow male was found (M. Reichard, unpublished data). To date, it is unknown whether the colour morphs are (or are associated with) adaptive traits driving the geographic patterning of their distribution or whether their distribution is the result of genetic drift and has no adaptive value. Importantly, red and yellow reflectance may provide an advantage in different visual environments: while the red part of the spectrum is more visible in clear water, the short wavelength of red colour is rapidly absorbed in a turbid light environment, in which yellow is more visible (Litjens, Quickenden & Freeman, 1999). This may have consequences for sexual selection *via* mate choice, as males with specific colouration may be more visible to females in particular light environment (for details on mate choice see Section III.6).

Fish skin is an organ undergoing continuous cellular and pigment turnover. Age-dependent changes in caudal-fin colouration are likely due to changes in gene expression occurring with age. Recently, the gene-expression patterns related to the two colour morphs were investigated by RNA-sequencing revealing that the yellow pigment cells express typical melanocyte genes whereas the red pigment cells show expression of myosin-associated genes (Ng'oma *et al.*, 2014*a*). Interestingly, the same genes are also differentially expressed in colour morphs of the Midas cichlid (Henning *et al.*, 2013). The yellow submarginal band with black marginal band is almost unique to *N. furzeri* (also observed in *N. virgatus* Chambers). However, the red morph is very common in the genus *Nothobranchius*, typically co-existing with blue (rather than yellow) male morphs within a species (Wildekamp, 2004).

Female *Nothobranchius* are always smaller than males, their fins are translucent and body is pale brown. In *N. furzeri* and other species, females often possess iridescent scales. In some species, such as *N. orthonotus*, *N. kadleci* Reichard and *N. melanospilus* (Pfeffer), the female body may be pigmented with small dark dots. It is not clear yet

whether this pigmentation arises as a byproduct of male colouration (Sedláček, Baciaková, & Kratochvíl, 2014) or has any adaptive value.

(5) Sex determination and sex ratio variation in the wild

Sex determination in *N. furzeri* is strictly genetic, with the male as the heterogametic sex (Valenzano *et al.*, 2009). Genetic sex determination with male heterogamety, although more complex than in *N. furzeri*, is also described from *N. guentheri* (Ewulonu, Haas & Turner, 1985) and may therefore be widespread in *Nothobranchius*. Genetic sex determination with females as XX and males as XY results in a balanced primary sex ratio, with 50% males and 50% females. This is likely also the case in *N. furzeri*. However, in wild populations females typically dominate (Reichard *et al.*, 2009). The adult sex ratio is often female biased soon after maturation, but sex bias in wild *N. furzeri* increases from a mean ratio of 1 male to 2.7 females to 1:4.7 at the end of the wet season (Reichard *et al.*, 2014). Extreme population sex ratios such as 0:29, 1:43, 1:35 or 2:43 have been reported for *N. furzeri* in the wild; on the other hand, extremely male-biased populations are not known (Reichard *et al.*, 2014). Sampling bias can be excluded, as some sampled habitats were small enough to reasonably assume that the entire fish population was sampled (Reichard *et al.*, 2014). Comparable seasonal declines in male abundance were reported from populations of a Neotropical annual fish, *Austrofundulus reicherti* (Loureiro and García) (Passos *et al.*, 2014). Given the equal adult sex ratio in the laboratory, high male mortality is the most plausible explanation for a female-biased sex ratio in the wild. While two species sympatric to *N. furzeri*, *N. orthonotus* and *N. pienaari* Shidlovskiy, Watters & Wildekamp, show comparable adult sex-ratio biases as wild *N. furzeri*, *N. kadleci* (a sister species to *N. furzeri*) has an adult sex ratio of 1:1. This surprising result was consistent across several years and was not a geographic artifact; *N. orthonotus* and *N. pienaari* populations sympatric with *N. kadleci* had typical female-biased sex ratios (Reichard *et al.*, 2014). The reason for this difference remains unknown.

Two hypotheses have been suggested to explain increased male mortality in the wild. First, predation on males is significantly higher than in females. This situation is reported for many taxa with high degrees of sexual dimorphism (Promislow, Montgomerie & Martin, 1992), including many fish (Haas, 1976*b*; Rosenthal *et al.*, 2001). Conspicuous male colouration may make them more visible to visually oriented predators such as birds (Haas, 1976*b*). Increased male mobility (Haas, 1976*b*) associated with mate searching and their larger size may further elevate the risk of predation by large belostomid hemipterans (Tobler, Schlupp & Plath, 2007; Tobler, Franssen & Plath, 2008), common predators in temporary savannah pools (Reichard *et al.*, 2014). In addition, males compete aggressively for access to females (Polačik & Reichard, 2011) and male competition for mates may also contribute significantly to male mortality (Polačik & Podrabsky, 2015).

(6) Reproductive behaviour, mating system and sexual selection

All *Nothobranchius* species have a polygynandrous mating system. Males and females often change their partner for each spawning act, although one pair often completes several (2–8) ovipositions in quick succession, without changing partners (Haas, 1976*a*). Only a single egg is laid during each oviposition event. Spawning bouts are repeated during the day and each *N. furzeri* female typically lays 20–50 eggs per day. The number of eggs produced can be largely modified by available resources and depends on female size and age (Blažek *et al.*, 2013). In young, actively growing females, or in suboptimal conditions, less than 10 eggs may be produced daily (Blažek *et al.*, 2013; Vrtílek & Reichard, 2015). With abundant food, a spawning of up to 291 eggs was recorded in *N. furzeri* and even 583 eggs in the closely related *N. kadleci* (Blažek *et al.*, 2013). Daytime fecundity in wild populations, as recorded by dissection of field-collected females, is comparable to laboratory populations, with a fecundity of 20–50 eggs per day (M. Vrtílek & M. Reichard, in preparation).

Males are constantly ready to mate and actively seek females to initiate spawning. The *Nothobranchius* courtship sequence is depauperate compared to other killifish (Passos *et al.*, 2013*b*). A detailed description of *N. guentheri* reproductive behaviour is given by Haas (1976*a*). Below, we provide a summary of the main behavioural sequence of *N. furzeri* based on Haas (1976*a*), with modifications pertinent to *N. furzeri* from Polačik & Reichard (2011) and derived from our unpublished observations.

Males establish a hierarchy based on male size (Polačik & Reichard, 2009). Males are active and explore the habitat in search of females, with dominant males controlling the largest areas. While males are not strictly territorial, specific parts of the pools are used as spawning arenas (conceivably due to a superior substrate for egg survival) and are likely controlled by dominant males. Upon encounter with a female, males display their lateral side with the dorsal and anal fins partly folded and perform a jerky gradual approach to attract the female's attention. Receptive females respond to males by allowing the male to approach and make physical contact. Unreceptive females flee, but may be followed by the male. The male clasps the female using his dorsal and anal fins, and the female places her anal fin close to the substrate. After cessation of movement for 1–3 s, the pair performs a rapid jerking movement during which a single egg is laid on the substrate. Egg spawning is associated with disturbance of the sediment and can be clearly recognized. This spawning action can be repeated following a renewed clasp between the male and female (Haas, 1976*a*).

Eggs are laid in the sediment and the jerking movements during oviposition likely help to cover the eggs with sediment. In captivity, sand, peat moss, coconut fibre or their combination are widely used for oviposition. These substrates are accepted by fish for oviposition and widely used for embryo development. However, the presence of a substrate is not required for oviposition, and captive *N. furzeri* successfully spawn in zebrafish breeding boxes where

no substrate is present; after fertilization, eggs fall through a mesh into a chamber which prevents their consumption by adult fish. Eggs are typically incubated on a wet substrate or in liquid culture in autoclaved tank water (Genade, 2005; Valenzano *et al.*, 2009; Blažek *et al.*, 2013).

Females are capable of making active mate choices and may approach a male or escape. At inter-population level, experimental evidence showed that *Nothobranchius korthausae* (Meinken) females preferentially associate with males that perform the most vigorous courtship. These females were physically isolated from males by a barrier but visual, olfactory and audio communication with males was possible (Reichard & Polačik, 2010). Nothing is currently known regarding signalling types other than visual signalling in *Nothobranchius* mate choice but in other fish taxa olfactory and auditory cues play important roles. Chemical communication occurs within a reproductive context in Neotropical annual killifish (Passos *et al.*, 2013*a*) and may play a role in *Nothobranchius* communication as well.

There have been no investigations of mate choice in *N. furzeri* and little is known about female preference for male colour morphs. In a single study on *N. korthausae*, females from a population with exclusive occurrence of red males preferred their sympatric (red) males. No colour preference was detected in a population with exclusive occurrence of yellow males (Reichard & Polačik, 2010). This study included fish from two geographically distinct populations so this outcome may be influenced by other, non-visual cues. Experiments with a population where males of both colour morphs co-occur in sympatry must be used to reveal female preference for male colour. Such study is possible in *N. furzeri* where coexistence of both male colour morphs is common. Recent pilot experiments did not detect any consistent preference for male colour in female *N. furzeri* (M. Polačik & M. Reichard, unpublished observations).

In competitive situations, dominant males tend to spawn more: they patrol larger areas and make more contacts with females. In addition, they may actively interrupt the spawning of other males (Haas, 1976*a*). In *N. korthausae*, there is no benefit (or cost) to females of spawning with dominant males in terms of fertilization success or hatching success (Polačik & Reichard, 2009; Reichard & Polačik, 2010) and females lay the same number of eggs regardless of male dominance status, at least under experimental conditions. The situation may be considerably different in the wild where dominant males may interfere with the spawning of other males.

Dominant males have the highest hue and brightness, suggesting that male colouration is sexually selected. In contrast to other fish, including the related pupfish (*Cyprinodon* spp.) (Kodric-Brown, 1977), male colouration is resistant to abrupt changes in behavioural context and maximum colour intensity is retained throughout the day (Haas, 1976*b*), with only minor changes in intensity during social interactions. Long-term changes in male colouration are associated with individual condition and health and male colouration also responds to the visual environment. Importantly, male colour fades with age in older adults (Lucas-Sánchez *et al.*, 2011). At an intra-population level, female *N. guentheri* are preferentially associated with brighter males (Haas, 1976*b*). In the wild, male colouration is strikingly brighter in habitats with clear water compared to turbid habitats.

In conclusion, male colouration may be sexually selected due to its importance in signalling dominance among males (intra-sexual selection) as well as in female choice (inter-sexual selection). Male colouration is species-specific and conveys species identity (Haas, 1976*b*). This will lead to reinforcement of species-specific colouration details, especially in sympatric species, and constrains selection on colour patterns. Male visibility to females, also described as 'sensory bias' or 'receiver bias' (Endler, 1980), often plays a significant role in sexual selection (Ryan, 1998). Red and yellow are of relatively long wavelengths compared to other parts of the visible spectrum and therefore are less absorbed in turbid waters (Litjens *et al.*, 1999), a typical habitat of *N. furzeri*. This is in contrast to the relatively rapid absorption of the blue part of the spectrum (short wavelengths); blue male forms are known from several *Nothobranchius* species, although none of these coexist with *N. furzeri* in turbid waters. Consequently, the strongest selection on bright male colouration may come from sensory exploitation by males making themselves most visible to females (receiver bias).

IV. LIFE CYCLE AND EMBRYONIC DEVELOPMENT

An annual life history and the ability of embryos to enter diapause is likely to be an ancient character state that has been lost several times during the evolutionary history of African and Neotropical killifish clades (Murphy & Collier, 1997; Hrbek & Larson, 1999). It is present in the basal taxa of Neotropical killifish such as *Millerichthys robustus* (Miller & Hubbs) (Domínguez-Castanedo, Mosqueda-Cabrera & Valdesalici, 2013) and intriguingly, prolonged developmental arrest similar to diapause III was experimentally induced in non-annual Neotropical killifishes (Varela-Lasheras & Van Dooren, 2014) demonstrating plasticity of embryonic developmental trajectories in the killifish clade.

Annual species from environments with a shorter rainy season spend the major part of their life cycle in diapause. For example, the average duration of *N. furzeri* habitats was only 75 days in one season (Terzibasi Tozzini *et al.*, 2013). Under these conditions, embryos are expected to spend almost 10 months in diapause. It is known that some embryos can survive more than a year in diapause in captivity: some *N. furzeri* eggs hatched successfully after 3 years of storage (A. Cellerino & M. Reichard, unpublished data). Multi-annual diapause may represent a bet-hedging strategy to survive particularly dry seasons when habitats are flooded for very short periods (Polačik *et al.*, 2014*a*) or not at all.

The embryonic development of annual fish was described in a series of three papers (Wourms, 1972*a,b,c*). A detailed description of developmental stages was provided for the

South American species *Austrofundulus myersi* Dahl, which produces large and transparent eggs; these stages are broadly applicable also to *Nothobranchius*. This description refers to embryos kept in liquid incubation to allow microscopic observations. It is possible that development in a natural substrate might differ in some respects from the described developmental pattern.

The early phases of embryonic development (cleavage) of *Nothobranchius* are characterized by very slow segmentation compared to *Danio rerio* (Hamilton), *Oryzias latipes* Temminck & Schlegel and non-annual cyprinodonts such as *Aphyosemion*. In teleosts, synchronous divisions and absence of the G1 and G2 phases characterize the cell cycle during the cleavage phase, so that cells proceed directly from the S to M phase (Graham & Morgna, 1966). Accordingly, all non-annual killifish species exhibit an average cell cycle length during cleavage of 20–45 min. By contrast, *N. furzeri* has a cleavage time of 75 min in the absence of a G1 phase. This retardation of the cell cycle is also observed in South American annual killifish as well as in the annual genus *Callopanchax* from West Africa with cell cycle lengths of 70–100 min (Dolfi, Ripa & Cellerino, 2014). This may result from the loss of an evolutionary constraint, since fertilized eggs of annual killifish are less subject to predation and hence may not be under selective pressure for rapid development.

A peculiar characteristic of annual fish eggs is the dispersed phase. At the end of the blastula stage, two cell types are produced. Cells committed to forming the enveloping layer migrate as a uniform sheet, and dispersed macroblastomeres migrate under this sheet. At the end of epiboly, there is a phase of morphogenetic stasis. During this phase, there is no evidence of a multicellular organizing centre and the macroblastomeres are under cell cycle arrest and migrate apparently randomly (Dolfi *et al.,* 2014). This phase has a fixed duration, but may be prolonged under unfavourable conditions and is also named diapause I. At the end of the dispersed phase, the macroblastomeres re-aggregate to form the embryonic axis. In *N. furzeri* (at 26°C), epiboly is completed in 48 h and the dispersed phase lasts about 5 days, so that the embryonic axis appears only after about 1 week. After re-aggregation, the neural keel is formed and then somitogenesis and morphogenesis of the nervous system proceed until the embryo enters diapause II. At this stage, the number of somites is fixed (32 in *Austrofundulus myersi*), the heart is tubular and contractile, the major divisions of the encephalon are present and so are the optic cups with the lens. The duration of diapause II is highly variable, from 2 days to up to 3 years. Temperature plays a major role in its regulation; embryos incubated at 28°C skip diapause and can hatch 12 days after fertilization. After diapause II, the embryo proceeds to complete development. South American annual killifish have an obligatory diapause III at the end of development (Berois *et al.,* 2014), where the embryo is completely formed but metabolism is inhibited, while *N. furzeri* does not show diapause III under standard incubation conditions.

Hatching is a critical process for survival, and during this process annual killifish are confronted by a very hard chorion to digest. In captive conditions, the timing of wetting of the eggs is critical for successful hatching; overincubation will lead to hatching failure or weakened juveniles incapable of swimming. In laboratory conditions, hatching can be induced by hypoxia (Levels *et al.,* 1986). Control of diapause and hatching is of paramount importance for natural populations but little is known about the environmental control of development and hatching in the natural environment.

Several authors (Podrabsky & Hand, 1999, 2000; Podrabsky, Carpenter & Hand, 2001; Duerr & Podrabsky, 2010) have studied the physiology of annual killifish embryos in the South American species *Austrofundulus limnaeus* Schultz. *A. limnaeus* originates from the Venezuelan llanos region with climatic patterns similar to those of East Africa and these findings could be relevant to *Nothobranchius*. Embryos of annual killifish are resistant to desiccation, as the egg envelope is composed of protein fibrils with characteristics of amyloid fibrils and is highly impermeable, significantly reducing water loss (Podrabsky *et al.,* 2001). Annual killifish embryos are also extremely resistant to hypoxia when in diapause II, with median survival in 0% O_2 of about 60 days (Podrabsky *et al.,* 2007). A number of physiological processes allow survival of the diapausing embryos. Oxygen consumption and protein synthesis are decreased by about 90% (Levels *et al.,* 1986; Podrabsky & Hand, 1999, 2000) and energy demand and production are reduced with increased AMP concentration corresponding to increased anaerobic metabolism. Diapause is associated with remodelling of mitochondrial physiology: mitochondria isolated from embryos arrested in diapause II are not poised to produce ATP, but rather to shuttle carbon and electrons through the Krebs cycle while minimizing the generation of a proton motive force (Duerr & Podrabsky, 2010). This likely represents a protective response to minimize the production of reactive oxygen species and avoid oxidative stress.

The duration of diapause II can be very variable and even absent ('skippers'). In *N. furzeri*, diapause II is always skipped if eggs are incubated in liquid medium at high temperature (28°C) and under these conditions, embryonic development can be completed in less than 2 weeks (Valenzano, Sharp & Brunet, 2011). A small fraction of skippers is observed, however, even in conditions favouring diapause (Polačik *et al.,* 2014*a*). This phenomenon is observed also in the inbred strain GRZ and therefore is not dependent on genetic heterogeneity.

The probability that an embryo enters diapause depends on maternal age and the number of eggs the female has already produced (Podrabsky, Garrett & Kohl, 2010). However, entrance and departure from diapause, especially diapause II, appears to be a largely stochastic process. Demographic and environmental factors only modify the likelihood and proportion of embryos entering diapause. Heterogeneity in the pace of embryo development could in theory provide a selective advantage in response to erratic rainfall. Possibly, 'skippers' are embryos that can hatch in the wild within a single rainy season in case a second flooding of the habitat occurs (Polačik *et al.,* 2014*a*). On the other hand, embryos

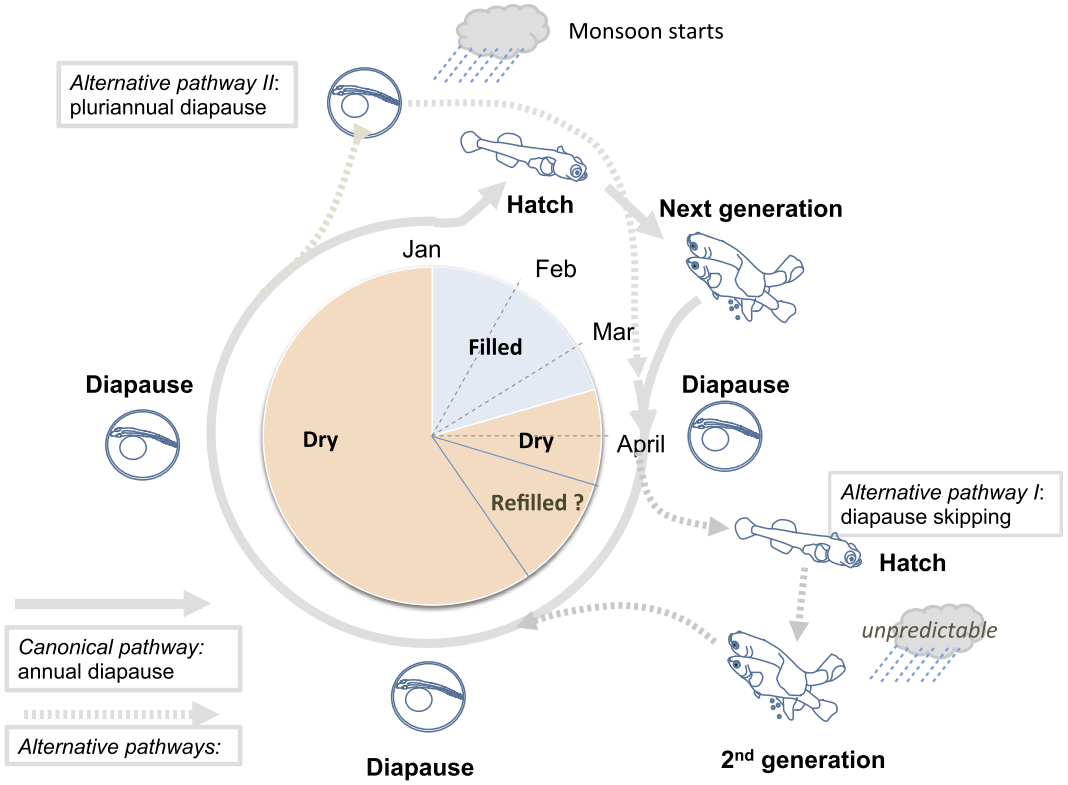

Fig. 3. Schematic representation of the *Nothobranchius* life cycle, including alternative developmental pathways. After flooding of the habitats (January), eggs hatch and the juveniles develop rapidly to reach sexual maturity. Habitat duration is estimated to be 2–3 months (March–April) and eggs remain in diapause for about 10 months (canonical developmental pathway, solid arrows). Some embryos skip diapause and proceed through direct development; these hatch if the habitat is re-filled again, during the same rainy season ('skippers', alternative pathway I, dashed arrows) – if the habitat is not re-filled they die. Some eggs remain in diapause for longer than 1 year and survive occasional dry years with no flooding of the habitat (pluriannual diapause, alternative pathway II, dashed arrows). Note that seasonal dynamics is scaled for *N. furzeri* and would differ in other *Nothobranchius* species.

that remain in diapause for more than a year may still hatch after a sequence of dry years with no flooding (Fig. 3).

After hatching, *Nothobranchius* show life-history adaptations expected for organisms form ephemeral habitats. Juveniles are immediately able to feed actively upon hatching. Under optimal laboratory conditions *N. furzeri* show rapid juvenile growth: the fastest maturation observed in a vertebrate with a typical duration of 3–4 weeks, and one recorded case of 18 days from hatching to sexual maturity (Blažek *et al.*, 2013).

V. POPULATION STRUCTURE

(1) Age structure

All individuals of *N. furzeri* co-occurring in the same pool are normally of the same age. In some wild populations, the age of individuals may vary. In that case, it is possible that either hatching takes a few days or, depending on the topography of the area, several depressions within a pool are flooded consecutively at different times (Polačik *et al.*, 2011). *N. furzeri* and other sympatric *Nothobranchius* species hatched between the last 2 weeks of December and the first week of January in two study years (2008 and 2009; Polačik *et al.*, 2011). This hatching date does not coincide with the start of the rainy season (October to November), but is in agreement with the season of major cyclones coming from the Indian Ocean, characterized by considerable precipitation over large areas of southern and central Mozambique (National Weather Service, 2014). Hence, the first rains may provide the environmental cue to end developmental diapause, likely through changes in gas partial pressure (e.g. hypoxia) and/or soil humidity, leaving fish at a fully developed stage capable of hatching within minutes after pool flooding, similarly to their rapid hatching in captivity (Genade *et al.*, 2005). It is unknown, however, whether hatching is similarly synchronized in other parts of the *Nothobranchius* range. Unlike intertropical regions with two rainy seasons, Southern Mozambique (where *N. furzeri* is found) is on the Tropic of Capricorn and has a single rainy season.

The age structure of *Nothobranchius* populations can be assessed through the determination of the number of daily increments in otoliths, small calcium carbonate structures located in fish inner ear. Comparison between otolith-determined age and body size revealed that individual- and population-specific factors such as social

interactions, resource availability or density determine body growth rates. Therefore, body size, *per se*, is not a reliable proxy for age (Polačik *et al.*, 2011).

(2) Genetic structure

An initial population genetic and phylogeographic analysis was carried out across the entire *N. furzeri* range (Bartáková *et al.*, 2013). Each savannah pool consists of panmictic, genetically homogenous populations of *N. furzeri*. There is no obvious intra-population segregation, for example associated with different male colour morphs. Populations are highly structured at a landscape scale and even adjacent pools contain genetically distinct populations. The same strong structuring was found for three other species: *N. orthonotus*, *N. kadleci* and *N. pienaari* (Bartáková, 2013). There is a clear pattern of isolation by distance, i.e. genetic differentiation between populations increases with geographical distance. In addition, two lineages – corresponding to the Chefu and Limpopo drainage systems – show a deep divergence, and a further divergence is observed between the south and north bank of the Limpopo defining three distinct genetic and geographic clades. Conventional mutation-rate-based timing places the oldest intra-specific lineage divergences prior to the Pleistocene (>2 Mya) (Dorn *et al.*, 2011; Bartáková *et al.*, 2013), although dating based on secondary calibration (derived from mutation-rate estimates in related taxa) suggests considerably younger divergence (0.5–1 Mya) (Dorn *et al.*, 2014). Irrespective of the exact timing, inter-population differences are high and gene flow between major intra-specific lineages is extremely limited. A putative suture zone has been described in a relatively flat area, where marginal populations of two deeply diverged lineages occur within 10 km, yet remain separate ($F_{st} = 0.128$). The same two lineages co-occur in at least one population in another part of their contact zone and nuclear genetic markers (microsatellites) indicate no reproductive barrier (Bartáková *et al.*, 2013). In the future, the use of genome-wide markers should reveal greater details of the population structure within and among populations.

(3) Dispersal and colonization

How *N. furzeri*, or any other *Nothobranchius* species, disperse and colonize new habitats is currently unknown. Adult or juvenile fish may be swept from their natal pools during extensive flooding and settle in a neighbouring pool. Alternatively, the eggs may be transported between savannah pools on the skin of large herbivores that use the pools for mud bathing and drinking, similarly to the eggs of macroinvertebrates (Vanschoenwinkel *et al.*, 2011), or entrained in mud on birds' feet. Finally, it is possible that changes in river morphology enable apparent river channel crossings. Each hypothesis creates a different set of predictions regarding population connectivity and their genetic structure. The river channels are predicted to have minor or major effects on phylogeographic pattern, and upstream dispersal is unlikely if flooding is involved. Importantly, these hypotheses are not mutually exclusive and fish may disperse *via* any of their combinations. The currently available data are not entirely consistent with either dispersal mode. Both rivers and river basins play important roles in structuring *Nothobranchius* communities (Dorn *et al.*, 2011) and significant migration to higher altitudes was detected (Bartáková *et al.*, 2013).

VI. DIFFERENCES BETWEEN WILD AND LABORATORY CONDITIONS

For any experimental work with captive organisms, caution should be taken when extrapolating results to the context of natural populations. Captive conditions vary greatly from those in the wild and important factors such as stress level, diet composition, predator absence or a lack of physical exercise may affect experimental outcomes. While such differences are unavoidable and insignificant for comparisons within and among different captive strains and treatments, their identification should help to generalize conclusions, put them into evolutionary and natural-history perspectives and frame their interpretation.

Food availability and diversity of prey, ambient temperature, individual interactions and sex differences in survival are the most obvious differences between wild and captive populations of *N. furzeri*, as well as other *Nothobranchius* species. Food availability in the laboratory is often discontinuous during the day, while wild fish are likely to forage throughout the day. Taxonomically and nutritionally diverse food is consumed in the wild (Polačik & Reichard, 2010) compared to the uniform diet of chironomids and *Artemia* spp. used almost exclusively in captivity. Commercial batches of chironomids also vary in quality due to variable amounts of indigestible debris and the presence of decomposed food. *Artemia* nauplii are a balanced food source (Sorgeloos, Dhert & Candreva, 2001) and are likely a good substitute for the natural diet. Importantly, an adult diet of chironomid larvae may induce liver steatosis in male fish (Di Cicco *et al.*, 2011). Inclusion of *Daphnia* spp. or other crustaceans in the diet of captive adult *N. furzeri* is possible, but any diet alteration decreases the reproducibility of experimental results across laboratories. Diet, food quantity and feeding frequency can modulate lifespan and ageing-related phenotypes in *N. furzeri* (Terzibasi *et al.*, 2009) as well as in other animals (Weindruch, 1996). *N. furzeri* can be conditioned to feed on commercial pellets. The development of a specific artificial food that meets the dietary requirements of *N. furzeri* would help to standardize laboratory conditions; studies in this direction are currently ongoing.

Water temperature fluctuates diurnally in the wild at amplitudes of up to 15°C (Reichard *et al.*, 2009) and natural temperature fluctuations are known to induce prominent changes in gene expression patterns in Neotropical annual killifish (Podrabsky & Somero, 2004). While temperature fluctuation is buffered in larger and deeper habitats, *N. furzeri* often inhabit shallow pools and are exposed to dramatic fluctuations in ambient temperature. Water temperatures

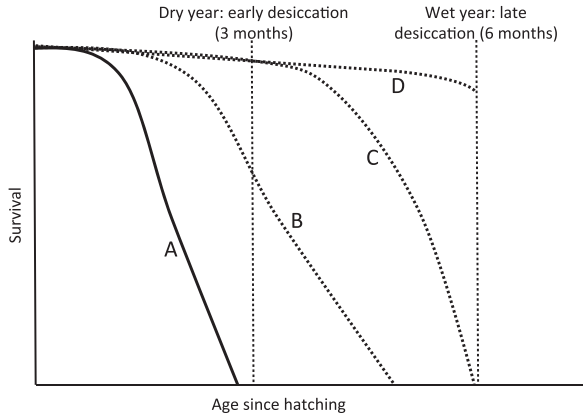

Fig. 4. Hypothetical effect of condition-dependent and condition-independent mortality on the survival of *Nothobranchius*. In population A, ageing is rapid and all individuals typically die before habitat desiccation. In this case, mortality may be strongly condition-dependent if driven by external factors (e.g. predation). In population D, mortality is relatively low until habitat desiccation and condition-dependence has, therefore, the most limited role. Populations B and C will have a component of condition-dependence, but its relative importance varies between generations; in dry years it is relatively low, but it is likely to be high in humid years since most mortality is not associated with desiccation but condition-dependent survival.

up to 37°C have been recorded frequently at the peak of the *N. furzeri* reproductive season (Reichard *et al.*, 2009), while it may decrease to 14°C in July, at the end of *N. furzeri* adult life in wild populations (M. Reichard, unpublished data). In the laboratory, water temperature is normally kept stable, with consequences for individual physiology, including gene expression patterns. Mimicking environmental fluctuation, however, will be inconsistent with strict laboratory protocols to ensure reproducibility.

Social structure in the laboratory populations is relatively stable as each individual encounters fewer opponents and sexual partners than in the wild. However, the frequency of individual encounters does not always differ dramatically between captive and wild populations. Wild populations vary greatly in their density and the density of captive fish of 30 individuals per m^2 may be close to high natural densities. Sources of natural mortality, another key difference between captive and natural populations, are discussed elsewhere (Fig. 4). Perhaps the most striking difference is the selective mortality of wild males, not observed in the laboratory (Reichard *et al.*, 2014).

VII. *NOTHOBRANCHIUS* AS A MODEL FOR STUDIES OF THE EVOLUTION OF AGEING AND LIFE-HISTORY TRAITS

(1) Condition-dependent and condition-independent sources of mortality

Ageing has been suggested to evolve as a trade-off with early investment in response to patterns of adult extrinsic mortality. Higher *extrinsic* mortality (mortality related to external factors such as predation) means that fewer individuals survive to reproduce at later ages, leading to weakening of selection for increased longevity. Therefore, high extrinsic mortality would result in rapid and dramatic ageing phenotypes as postulated by the classical theories (Medawar, 1952; Williams, 1957; Kirkwood, 1977). Rapid ageing is thus the result of higher intrinsic mortality, i.e. more rapid deterioration of vital functions, even in benign (e.g. laboratory) conditions where individuals are shielded from extrinsic mortality.

The relationship between extrinsic mortality and ageing, however, is likely more complex. For example, traditional evolutionary theories of ageing (Medawar, 1952; Williams, 1957; Kirkwood, 1977) do not consider extrinsic mortality to be condition dependent (Chen & Maklakov, 2012), i.e. that individuals vary in their likelihood of dying due to their genetic background manifested as differential immune responses, predator avoidance or ability to cope with other external stressors. While standard theories of ageing do not consider such individual differences important, such a distinction is in fact a reasonable assumption. Individual variance in survival-enhancing traits is a major source of Darwinian fitness (lifetime reproductive success) (Darwin, 1859). A positive genetic correlation between longevity and survival-enhancing traits is predicted to arise under challenging environmental conditions (Williams & Day, 2003). Strong condition-dependent extrinsic mortality may indeed lead to the evolution of longer (rather than shorter) lifespan (Chen & Maklakov, 2012), challenging a major paradigm of current theories of senescence. Under natural conditions, extrinsic mortality likely combines both condition-dependent and condition-independent components.

Inter-sexual differences in mortality rates in wild populations, not observed in the laboratory, are linked to extrinsic mortality. Increased male mortality in the wild clearly arises from extrinsic mortality, most likely predation (Haas, 1976*b*; Reichard *et al.*, 2014). Given the difference between protected (laboratory) and unprotected (wild) environments, such mortality is clearly condition-dependent. Therefore, within a population, the sexes may differ in the relative importance of intrinsic and extrinsic mortality, making inter-sexual comparison in ageing and survival a possible avenue for ecological tests of survival.

(2) Natural gradient of life expectancy

Populations of *Nothobranchius* from a natural gradient of aridity are suitable for testing predictions from evolutionary theories of ageing, populations are expected to vary in their intrinsic mortality rates. Given large differences in habitat duration, fish from more humid parts of the range are exposed for longer to forces of natural selection on survival, and their mortality will have a strong component of condition-dependence (Fig. 4: population C in humid year). In the dry part of their range, habitats desiccate more rapidly and mortality may be more condition-independent, coincident with the catastrophic disappearance of the habitat

before condition-dependent survival affected most individuals (Fig. 4: population C in dry year). These conditions may mean that individuals experience selection pressures for rapid development and high fecundity. Each scenario, and their combination, leads to different predictions (Fig. 4).

A first attempt to compare lifespan and ageing across the gradient of life expectancy used inter-specific comparisons and suggested strong differences in ageing and lifespan (Terzibasi Tozzini *et al.*, 2013). Under standardized captive conditions, median survival in three *N. furzeri* populations (from relatively arid regions) was 17.5–29 weeks, while a closely related species, *Nothobranchius kuhntae* (Ahl), from a more humid region had a median lifespan of 42–47 weeks. Similar contrasts were observed for maximum lifespan (defined as 10% survivorship): 33–40 weeks in *N. furzeri* and 50–57 weeks in *N. kuhntae*. The same trend was evident also in a second pair of species: a population of *N. pienaari* from the dry region had a median lifespan of 33 weeks, a population from an intermediate region 41 weeks, and two populations of its sister species, *N. rachovii*, from the humid region of the gradient had a median lifespan of 40–55 weeks. Maximum lifespan corroborated these findings: 43, 55 and 63–65 weeks, respectively (Terzibasi Tozzini *et al.*, 2013). Deposition of lipofuscin (a pigment that accumulates with increasing age and can be used as a marker of ageing rate) can be assessed in wild fish; short-lived populations always contained more lipofuscin in their livers and brains at a given age (Terzibasi Tozzini *et al.*, 2013), suggesting that differences in lifespan were associated with differential (intrinsic) ageing rates both under laboratory conditions and in the wild. The next challenge is to test the evolution of lifespan and ageing along the gradient at an intra-specific level.

(3) Coevolution between ageing and life-history traits

The complexity of the ageing process and selective forces acting on longevity is underpinned by evolutionary trade-offs between ageing and other life-history traits. General life-history theory (Stearns, 1992) predicts that individuals at high risk of extrinsic mortality (i.e. harsh and unpredictable environments in the case of *N. furzeri*) should be selected for high levels of resource allocation to current reproduction to maximize their lifetime fitness. Therefore, more rapid growth and earlier maturation (at a smaller size and age) are predicted in *N. furzeri* populations from dry parts of their range. Additionally, current reproduction should be preferred over allocation to further growth and self-maintenance, leading to a higher relative fecundity. These predictions are being tested using *N. furzeri* populations from dry and wet regions of the range, meta-replicated for similar contrasts using three other related species from the same area (*N. kadleci*, *N. orthonotus*, *N. pienaari*) (R. Blažek & M. Reichard, unpublished data). Since *Nothobranchius* have non-overlapping generations, they are extremely well suited to investigations of population dynamics and the demography of ageing in the wild.

(4) Reaction norms of life-history traits

A reaction norm is an interval between extremes of plasticity in genotype expression; it sets limits to the gene by environment interaction. Phenotypic plasticity, the capacity of an individual genotype to produce variable phenotypes in different environmental conditions, is a powerful strategy to fine-tuning individual traits to current conditions. Phenotypic expression of a trait can be irreversible (such as time of sexual maturation) or dependent on current conditions (batch fecundity, growth rate).

Since *N. furzeri* inhabits exceptionally unpredictable habitats, it is expected to have evolved highly plastic responses to environmental fluctuations. Indeed, key life-history traits vary considerably in *N. furzeri*, depending on current conditions. For example, sexual maturation can be achieved in only 18 days when conditions are optimal (Blažek *et al.*, 2013), but delayed until the age of 5 weeks under suboptimal conditions (Graf, Cellerino & Englert, 2010). Daily egg production can vary from none to over 200 eggs. Egg size also differs among females and among conditions; egg diameter along the longest axis varies from 1.1 to 1.3 mm. Allocation between growth and reproduction is exceptionally flexible and fish are capable of full compensatory growth after improvement in conditions (Vrtílek & Reichard, 2015).

Wide reaction norms in *N. furzeri* mean that demographic parameters such as population density and food availability may mask differences in mean trait values among populations in the wild. However, controlled standardized conditions, where variation in environmental and demographic factors is minimized (i.e. common garden experimental conditions), have the power to detect signatures of selection on particular life-history traits. It remains to be analysed whether large inter-annual variability favours generalized evolution of broad reaction norms, or whether it can push trait mean values towards local optima.

VIII. THE AGEING PHENOTYPE

Median lifespan of *N. furzeri* in captivity can be as short as 3 months for the GRZ inbred strain (Valdesalici & Cellerino, 2003). Records of captive lifespan for recently wild-derived strains are all in the order of at least 6–8 months and do not replicate the extreme GRZ phenotype, including two strains collected at the Zimbabwe/Mozambique border, within 25 km of the original collection site of GRZ (Terzibasi Tozzini *et al.*, 2013; D. R. Valenzano, unpublished observations). These observations suggest that the short lifespan of the GRZ strain is an extreme phenotype even within *N. furzeri*. On the other hand, it can be excluded that the extremely short lifespan of GRZ is the result of a single recessive mutation; genetic mapping studies did not detect a single locus of major effect, suggesting the complex multigenic nature of this trait (Kirschner *et al.*, 2012; Ng'oma *et al.*, 2014*b*). This makes it highly unlikely that this phenotype is the result of a *de novo* mutation but instead must arise from

a combination of naturally occurring alleles. In addition, artificial selection in captivity may have targeted individuals with the most rapid growth and maturity, coinciding with a very short lifespan, as is the case for laboratory mice (Miller *et al.*, 2002). In the future, studies of quantitative trait loci (QTL) mapping combined with targeted re-sequencing of genomic regions from natural populations will reveal what genomic regions and genes differ between the GRZ strain and other more recently wild-derived strains.

It is important to highlight that captive lifespan is highly dependent on culture conditions such as water temperature and chemistry, food source and its availability, fish density, light intensity and water flow. Different experimental settings may result in different lifespans to those reported here.

(1) Integrative traits

Given the extremely short lifespan of *N. furzeri* compared to other vertebrates, a considerable fraction of early investigations in this species aimed at confirming that the short lifespan was a consequence of accelerated ageing. Old *N. furzeri* show a typical *habitus* of emaciation, spinal curvature, and reduced colouration in males (Fig. 5A; Table 2). Similar age-related phenotypes are known in *Danio rerio*, *Oryzias latipes*, *Poecilia reticulata* Peters and *Austrolebias belloti* (Gerhard *et al.*, 2002; Hatakeyama *et al.*, 2008). One peculiar trait is the continuous growth of the eye (Lucas-Sánchez *et al.*, 2011), so that old individuals have larger eyes (Fig. 5A). *N. korthausae* shows similar macroscopic age phenotypes as *N. furzeri*, although their onset is delayed, as this species can survive for up to 18 months (Lucas-Sánchez *et al.*, 2011; Baumgart *et al.*, 2015). Remarkably, the expression of these age-dependent phenotypes is rather variable across individuals of the same age; some individuals displaying the 'decrepit' phenotype may survive for months.

At a behavioural level, there is a generalized reduction in spontaneous locomotor activity, with older *N. furzeri* individuals spending less time exploring compared to young ones (Genade *et al.*, 2005). This reduced activity is also reported in *N. korthausae*, coupled to disruption of circadian rhythms (Lucas-Sánchez *et al.*, 2011). *N. furzeri* exhibit a significant impairment of learning performance with age, when tested using an active avoidance task. The performance of older individuals learning to associate a conditioned stimulus (light onset) with an unconditioned stimulus (water disturbance following light onset) was significantly lower than that of younger subjects (Valenzano *et al.*, 2006*b*; Terzibasi *et al.*, 2008).

Histopathological examinations have revealed the nature of age-related organic decay (Di Cicco *et al.*, 2011). *Post-mortem* analyses revealed lesions primarily in kidney (Fig. 5B–E), liver and heart. Kidneys undergo tubule dilatation and crystal deposition (Di Cicco *et al.*, 2011). Importantly, reduced renal function and nephrocalcinosis are also important aspects of the human geriatric phenotype (Silva, 2005*a*,*b*). The cardiac lesions included hypertrophy of the cardiomyocytes (Di Cicco *et al.*, 2011), another typical aspect of vertebrate ageing (Woodhead, 1984; Dai *et al.*, 2012). Kidney lesions

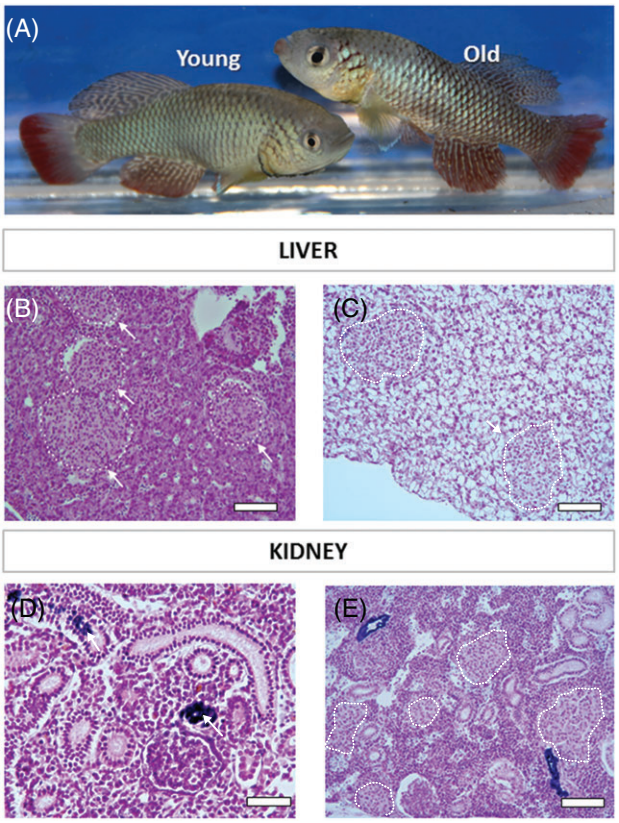

Fig. 5. Phenotypes associated with ageing in *Nothobranchius furzeri*. Macroscopic view and examples of the most common histopathological lesions. (A) Young and old males of the red morphotype. Note the larger eye, discolouration, frayed fins, and emaciation in the older individual. (B) Liver; hepatomas are indicated by white arrows and dotted lines. The liver parenchyma does not show steatosis in this section. (C) Liver; hepatocarcinomas are indicated by white arrows and dotted lines. Steatosis of the parenchyma is apparent. (D) Cephalic kidney; nephrocalcinosis indicated by calcium precipitates (white arrows) into dilatated tubules leading to tubular necrosis. (E) Cephalic kidney; neoplastic lesions are indicated by dotted lines. Calcium precipitates are also visible. Scale bars: (B, C, E) 200 µm; (D) 100 µm. Panels (B–E) are reproduced with permission from Di Cicco *et al.* (2011).

were similar to age-dependent lesions described previously in *P. reticulata* (Woodhead, Pond & Dailey, 1983). Clearly, *N. furzeri* suffer from a rapid and concerted ageing process that affects several organs and is similar to that of other vertebrates, including humans.

A point of particular interest is age-dependent neoplasia. Tumour onset is clearly age-associated in humans. Tumours are rare in teleost fish. However, a high incidence of spontaneous neoplasias was observed in the liver and kidney of *N. furzeri* (Di Cicco *et al.*, 2011). These were hepatomas and cellular hepatocarcinomas that were present in about 50% of subjects at median lifespan in both sexes. The onset of liver neoplasias was dependent on the longevity of the strain; it was accelerated in the very short-lived GRZ strain (12 weeks *versus* 6 months), while the same neoplasic lesions

Table 2. Conserved ageing phenotypes of *Nothobranchius* spp. and their occurrence in other model taxa

Description	Other species
Integrative phenotypes	
Spinal curvature[a, b]	Zebrafish[c], medaka[d],
Emaciation[a, b]	Zebrafish[c], medaka[d]
Disrupted circadian rhythm[b]	*Drosophila*, zebrafish[e], mammals
Reduced spontaneous activity[a, b]	*Drosophila*, mammals
Cognitive impairments[f]	*Drosophila*, zebrafish, mammals
Cellular phenotypes	
Apoptosis (liver)[g]	Medaka[h]
Lipofuscin (liver, brain, gills)[a, i, j]	Nearly universal
Cardiac hypertrophy[g]	Guppy[k], mammals
Kidney tubule dilatation[g]	Guppy[l]
Liver neoplasias[g]	Medaka (low incidence)[m]
Senescence-associated β-Gal[a, i, j]	Zebrafish[n], human[o]
Gliosis[p]	Mammals
Quiescence of neuronal stem cells[p]	Zebrafish[q], mammals
Molecular phenotypes	
Telomere shortening[r]	Medaka[d], human
Lipid peroxidation[s]	Nearly universal
Mitochondrial impairments[t]	Mammals

β-Gal, β-galactosidase.
[a]Genade *et al*. (2005).
[b]Lucas-Sánchez *et al.* (2011).
[c]Gerhard *et al*. (2002).
[d]Hatakeyama *et al*. (2008).
[e]Zhdanova *et al*. (2008).
[f]Valenzano *et al*. (2006*a*,*b*).
[g]Di Cicco *et al*. (2011).
[h]Ding *et al*. (2010).
[i]Hsu *et al*. (2008).
[j]Liu *et al*. (2012).
[k]Woodhead (1984).
[l]Woodhead *et al*. (1983).
[m]Masahito *et al*. (1989).
[n]Kishi *et al*. (2003).
[o]Dimri *et al*. (1995).
[p]Tozzini *et al*. (2012).
[q]Edelmann *et al*. (2013).
[r]Hartmann *et al*. (2009).
[s]Lucas-Sánchez *et al*. (2014).
[t]Hartmann *et al*. (2011).

were described in the longer-lived *N. guentheri* (Cooper *et al.*, 1983) at 12 months. Hepatomas and hepatocarcinomas were also described in ageing medaka (*Oryzias latipes*) females (but not males) at a low frequency (∼7%) (Masahito *et al.*, 1989).

A peculiar type of lesion was the accumulation of fat in the liver (steatosis; Fig. 5C) particularly in males (Di Cicco *et al.*, 2011). These lesions have no effect on lifespan as male and female survivorship is identical for laboratory-bred *N. furzeri* (Valenzano *et al.*, 2006*b*; Graf *et al.*, 2010; Kirschner *et al.*, 2012) and is likely linked to diet, as steatosis was seldom observed in wild animals (A. Cellerino & M. Reichard, unpublished data). Age-dependent liver lipid accumulation is also observed in captive mice (Park *et al.*, 2014).

(2) Cellular traits

Ageing at the cellular level was investigated in liver, skin and brain of several *Nothobranchius* species (Table 2). In the liver, age-dependent accumulation of lipofuscin is a highly reproducible phenotype (Valenzano *et al.*, 2006*a*; Terzibasi *et al.*, 2008, 2009; Liu *et al.*, 2012; Ng'oma *et al.*, 2014*b*) and age-dependent accumulation of lipofuscin is observed also in wild *Nothobranchius* spp. (Terzibasi Tozzini *et al.*, 2013). Lipofuscin, also known as 'age pigment', is an autofluorescent pigment that accumulates in the lysosome as a result of lipid peroxidation and accumulates with age in many organisms from nematodes to humans (see discussion in Ng'oma *et al.*, 2014b) and is an endpoint of analysis for intervention studies in *Nothobranchius* spp. (Valenzano *et al.*, 2006*a*; Hsu & Chiu, 2009; Terzibasi *et al.*, 2009; Yu & Li, 2012). Increased apoptosis with ageing is also observed in the liver of *N. furzeri* (Di Cicco *et al.*, 2011; Ng'oma *et al*., 2014*b*) and medaka (Ding *et al.*, 2010). Interestingly, age-dependent profiles of lipofuscin and apoptosis are different. Lipofuscin accumulates linearly with age whereas apoptotic rate shows a bell-shaped trajectory where the oldest animals (past median lifespan) show a reduced apoptotic rate as compared to individuals sampled around median lifespan (Ng'oma *et al.*, 2014*b*). This suggests that lipofuscin accumulates as a neutral marker of age while apoptosis is more related to individual phenotypic conditions: individuals with lower apoptotic rates are more likely to reach old age. Both lipofuscin and apoptotic rates are high in the GRZ strain (Ng'oma *et al.*, 2014*b*). A direct demonstration that lipofuscin and apoptotic rate are markers of different age-dependent processes comes from the analysis of F_2 hybrids between *N. furzeri* GRZ and *N. kadleci* where the two traits are uncorrelated and are controlled by different quantitative trait *loci* (Ng'oma *et al.*, 2014*b*).

In the skin, there is evidence that ageing is associated with accumulation of cells that have irreversibly lost the ability to enter the cell cycle (replicative senescence). Human senescent cells can be identified *in vivo* by expression of the histochemical marker β–Gal (β–galactosidase). Age-dependent increase of β–Gal staining was reported in *N. furzeri* and two other *Nothobranchius* species (Genade *et al.*, 2005; Hsu *et al.*, 2008; Liu *et al.*, 2012). Further evidence for cellular senescence is provided by age-dependent up-regulation of cell-cycle inhibitors such as cyclin-dependent kinase inhibitor 1A (*CDKN1A*, p21) and growth arrest and DNA damage (GADD45) family members (Graf *et al.*, 2013; Baumgart *et al.*, 2014)

The *N. furzeri* brain was studied with reference to its morphology and gene expression patterns (D'Angelo *et al.*, 2012, 2014; D'Angelo, 2013). The adult teleost brain is more similar to the embryonic than to the adult mammalian brain. Fish brains show extensive presence of neuronal stem cells of both neuroepithelial and radial glia nature throughout the rostro-caudal axis of the encephalon, they lack astroglia (the mature glia in mammalian brains), and all glia are radial, retaining neurogenic properties (Cuoghi & Mola, 2009). The major cellular phenotypes observed in ageing *N. furzeri* brains are: (*i*) dramatic reduction of stem cell activity (Tozzini *et al.*, 2012), which parallels the well-known age-dependent reduction of adult neurogenesis described in mammals (Kempermann, 2011); (*ii*) glial hypertrophy

(gliosis), visualized as over-expression of glial fibrillary acidic protein (GFAP) (Tozzini *et al.*, 2012), also a typical phenotypic response of mammalian glia to injury, neurodegenerative disease and ageing (Norton *et al.*, 1992; Bronson, Lipman & Harrison, 1993); (*iii*) neuronal degeneration, as measured by Fluoro-Jade B staining in *N. furzeri* and *N. guentheri* (Valenzano *et al.*, 2006*b*; Terzibasi *et al.*, 2009; Liu *et al.*, 2012); and (*iv*) accumulation of lipofuscin (Terzibasi *et al.*, 2009; Terzibasi Tozzini *et al.*, 2013). Finally, a potential loss of neurons was indirectly suggested in the brain of *N. guentheri* based on neurofilament labelling (Genade & Lang, 2013), although this result requires confirmation using different labelling techniques and stringent stereological methods (Schmitz & Hof, 2007).

(3) Molecular traits

At a molecular level, the major changes described in *Nothobranchius* species (Table 2) are: telomere erosion (despite somatic expression of telomerase activity) observed in three different species and multiple organs (Hsu *et al.*, 2008; Hartmann *et al.*, 2009; Liu *et al.*, 2012); reduced mitochondrial numbers (as assessed by mtDNA copy numbers) and function, especially in liver, muscle and brain (Hartmann *et al.*, 2011); reduction of peroxidation-sensitive polyunsaturated fatty acids (in *N. korthausae*) (Lucas-Sánchez *et al.*, 2011), and modulation of mitochondrial fatty acid profile (in *N. rachovii*), which is also consistent with increasing damage (peroxidation) to mitochondrial lipids (Lucas-Sánchez *et al.*, 2014). These results on fatty acid composition (and linked peroxidation) are also consistent with age-dependent accumulation of lipofuscin, a product of lipid peroxidation.

IX. GENOME-WIDE GENE EXPRESSION STUDIES ON AGEING

The introduction of next-generation sequencing technology has allowed genome-wide quantification of gene expression in *N. furzeri*, analysing both small non-coding RNAs as well as protein-coding genes.

(1) MicroRNAs

MicroRNAs are highly conserved, small non-coding RNAs that can be easily quantified even in the absence of a reference genome. Analysis of microRNAs in the ageing brain has revealed a down-regulation of oncogenic microRNAs and up-regulation of tumour suppressor microRNAs. In particular, age-regulated microRNAs are part of a gene regulatory network centred on the antagonistic actions of the prototypic tumour suppressor tumor protein p53 (*TP53*) and the prototypic oncogene v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) with up-regulated microRNAs showing positive interactions with *TP53* and negative interactions with *MYC* and down-regulated microRNAs showing the opposite pattern (Baumgart *et al.*, 2012). Based on these observations, microRNAs might be involved in controlling the neurogenic activity of neuronal stem cells. Indeed, when the expression of microRNAs in the brain of *N. furzeri* was studied using *in situ* hybridization, it was found that both the oncogenic microRNA cluster 17∼92 and the tumour suppressor microRNA miR-15a are expressed specifically in the adult neuronal stem cells (aNSC) niche, but with opposing dynamics; the former being down-regulated and miR-15a up-regulated upon ageing (Terzibasi Tozzini *et al.*, 2014). In addition, age-dependent regulation of microRNAs appeared conserved between *N. furzeri*, rodents and primates (Baumgart *et al.*, 2012). Interestingly, microRNA expression in the short-lived GRZ strain was severely deregulated and the pattern of expression of the short-lived GRZ strain at 5 weeks was reminiscent of that of the longer-lived MZM0-410 strain at 11 weeks, indicating that the expression profiles of these two strains diverge prior to sexual maturity (Baumgart *et al.*, 2012).

(2) Protein-coding genes

The transcriptome of *N. furzeri* was recently sequenced (Petzold *et al.*, 2013) and more than 19000 protein-coding genes with sequence homology to other vertebrate species were identified. This catalogue was used as a reference to quantify the expression of protein-coding genes in the ageing brain (Baumgart *et al.*, 2014). RNA expression was quantified at five age points in the longer-lived strain MZM0-410, corresponding to sexual maturity (5 weeks), young adults (12 weeks), adults (20 weeks, as defined by a decrease in growth rate), median lifespan (27 weeks) and old age (39 weeks, ∼30% survivorship). Comparison with human data sets revealed conserved age-related down-regulation of genes coding for proteins related to processing and turnover of RNA, translation and proteostasis with up-regulation of genes coding for ribosomal and lysosomal proteins, and down-regulation of genes coding for proteins involved in targeting to endoplasmic reticulum and nonsense-mediated RNA decay (Table 3). Further, ageing was characterized by conserved down-regulation of genes coding for protein folding, proteasome and spliceosomes. On a broader level, results from *N. furzeri* were compared in a meta-analysis of different human, primate and rodent tissues. Four up-regulated terms ('cytosolic ribosome', 'lysosome', 'negative regulation of apoptosis' and 'complement activation') and two down-regulated terms ('mitochondrion' and 'collagen') were shared (Baumgart *et al.*, 2014). These data emphasize that *N. furzeri* can be used to model human ageing.

Down-regulated genes differed in their temporal profiles: a decrease in expression of genes involved in neurogenesis and extracellular matrix was rapid, whereas it was progressive for genes coding for synaptic and axonal proteins (Baumgart *et al.*, 2014). This reflects a rapid decay of neurogenesis as opposed to a gradual decrease of neuronal function. A substantial proportion of differentially expressed genes (∼40%) showed inversion of their temporal profiles at the last time point: spliceosome- and proteasome-related genes

Table 3. Shared pathways in human and *N. furzeri* brain ageing

GO domains	Top categories
Over-represented among up-regulated genes	
GO: biological process	Translational elongation
GO: cellular component	Ribosome
	Lysosome
Over-represented among down-regulated genes	
GO: biological process	Cell cycle
	DNA damage
	Cellular response to stress
	Protein folding
	RNA processing
GO: cellular component	Nucleus
	Organelle
	Microtubule cytoskeleton
	Synapse/axon
	Mitochondrion
KEGG pathways	Proteasome
	RNA transport
	Spliceosome
	Nucleotide excision repair
	Protein processing in endoplasmic reticulum

Down- or up-regulated genes were classified according to their gene ontology (GO) as indicated by the International GO Consortium (www.geneontology.org). GO is a formal representation of the knowledge of the function of a specific gene. The biological process domain represents the knowledge concerning the involvement of a gene in a complex series of molecular events, whereas the cellular component domain represents the knowledge concerning the cellular localization of the gene product. In addition, where possible, genes were assigned to specific pathways according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG).
Adapted from Baumgart *et al.* (2014).

showed initial down-regulation and stress-response genes initial up-regulation. This may be explained by age-dependent selection for the most robust phenotypes, as animals measured at the last time points may be of high phenotypic quality, allowing them to reach old age (see also Section VIII.2). This analysis also showed prominent epigenetic remodelling with age: extensive regulation was detected for chromatin remodellers of the DNA (cytosine-5-)-methyltransferase (*DNMT*) and chromobox homolog (*CBX*) families as well as members of the polycomb complex and was mirrored by up-regulation of the tri-methylation of lysine of histone H3 (H3K27me3) epigenetic mark (Baumgart *et al.*, 2014).

Network analysis showed extensive co-regulation of cell cycle/DNA synthesis genes with the uncharacterized zinc-finger protein ZNF367 as a central hub. *In situ* hybridization showed that ZNF367 is expressed in neuronal stem cell niches of both embryonic zebrafish and adult *N. furzeri*. The same gene is also strongly down-regulated during ontogeny of the prefrontal cortex in humans (Colantuoni *et al.*, 2011; Baumgart *et al.*, 2014). Baumgart *et al.* (2014) not only revealed strong conservation in whole-genome transcript regulation between humans and *N. furzeri* but also, for the first time, exploited *N. furzeri* to identify new molecular targets of ageing.

X. *NOTHOBRANCHIUS* AS A MODEL FOR QUANTITATIVE GENETICS

Several species of the genus *Nothobranchius* display large, naturally evolved intra-specific phenotypic differences (Terzibasi *et al.*, 2008; Reichard *et al.*, 2009; Terzibasi Tozzini *et al.*, 2013) including colour morphs, behavioural traits such as spontaneous swimming and learning performance, as well as survival in captivity. This provides a great opportunity for genetic linkage studies aimed at identifying the genetic architecture underlying these traits, which could also help reveal their evolutionary history.

The analysis of a genetic cross between two *N. furzeri* strains, one from a yellow and the other from a red morph, showed that male-specific caudal colour differences have a simple genetic architecture and depend on a single genomic region, with yellow dominant over red (Valenzano *et al.*, 2009; Kirschner *et al.*, 2012). Current work aims to identify what gene and exact sequence (coding or regulatory) is causally associated to this macroscopic phenotypic change, and whether these regions are under selection.

Interestingly, both in the wild and in captivity, the yellow pigment can change with age in heterozygote adult males, with the yellow caudal fin colouration changing into red. Additionally, male fish of the yellow colour morph, unlike the red, have increased likelihood of developing pigment aberrations, including the development of black and red blotches in the caudal fin. It is not clear whether this possibly aberrant phenotype affects female preference, and whether it is associated to an increased risk of skin cancer.

In *N. furzeri* the male is the heterogametic and female the homogametic sex (Valenzano *et al.*, 2009). The male-specific Y chromosome is most likely non-degenerate and presents a region harbouring the sex-determining gene, where recombination with the X chromosome is suppressed, and two pseudoautosomal telomeric regions, where recombination with the X chromosome is allowed (Valenzano *et al.*, 2009). Differentiation of sex-determining mechanisms among different species provides an impenetrable barrier to hybridization and can therefore promote speciation events (Kitano *et al.*, 2009). Studies on the genetics of sex determination in different *Nothobranchius* species could shed light on diversification and speciation processes. Future work will further dissect the genetic architecture of the sex chromosomes in *N. furzeri*, to identify the master sex-determination gene, and to infer the time when this element became functional during the evolution of this species and how it differs from sex-determining systems present in other species of the genus *Nothobranchius*, such as *N. guentheri* (Ewulonu *et al.*, 1985).

In addition to opportunities to study the genetic architecture of Mendelian traits, *Nothobranchius* species provide a rare example of intra-specific diversification of ageing and survival phenotypes. Mapping QTL for captive survival in genetic crosses has shown that this species has a few genomic regions that contribute to the genetic variance for adult lifespan (Kirschner *et al.*, 2012) and for the rate of

lipofuscin deposition and apoptosis (Ng'oma *et al.*, 2014*b*). The identification of QTL for longevity and histological ageing traits in this species provides a unique opportunity to identify, in an unbiased way, genomic regions underlying natural differences in survival and ageing phenotypes among vertebrates.

XI. *NOTHOBRANCHIUS* AS AN EXPERIMENTAL MODEL

(1) Non-genetic interventions

Over recent years, different research groups have used several species of the genus *Nothobranchius* to test the effects of non-genetic manipulations on vertebrate survival and ageing-related phenotypes. These manipulations include changes in temperature, diet and drug administration. Changes in temperature modulate ageing in *C. elegans* and *D. melanogaster* (Mair *et al.*, 2003; Xiao *et al.*, 2013), as well as in mice (Conti *et al.*, 2006). *Nothobranchius furzeri* and *N. rachovii* ageing rate and overall survival in captivity can be modified by water temperature (Valenzano *et al.*, 2006*a*; Hsu & Chiu, 2009), with lower temperature increasing lifespan, slowing down the appearance of ageing biomarkers, reducing adult body size and delaying the age-dependent decline in spontaneous activity.

Modulation of diet intake in many model organisms, including *C. elegans*, *D. melanogaster* and rats, has proved an effective way to modulate ageing and survival (McCay, Crowell & Maynard, 1935; Mair *et al.*, 2003; Greer & Brunet, 2009). Dietary restriction by feeding every other day in inbred and wild-derived *N. furzeri* strains was effective in prolonging the survival of inbred strains and slowing down ageing, but had a mixed effect in wild-derived strains, increasing early mortality while prolonging maximum lifespan (Terzibasi *et al.*, 2009), similarly to results reported in mice (Weindruch, 1996). Different research groups have shown that in both *N. furzeri* and *N. guentheri*, the natural polyphenol resveratrol can slow down ageing and prolong lifespan (Valenzano *et al.*, 2006*b*; Yu & Li, 2012; Genade & Lang, 2013). Additionally, supplementation of melatonin to aged *N. korthausae* reduced age-dependent disruption of circadian rhythms (Lucas-Sánchez *et al.*, 2013). Combined with their short lifespan and the availability of a large set of ageing biomarkers, these results suggest that species of the genus *Nothobranchius* are excellent model systems to test non-genetic interventions and their effects on ageing and survival. In particular, these fish offer a great opportunity to study the effects of water-soluble drugs and feeding on vertebrate survival and ageing on a large scale.

(2) Transgenesis

The gold standard for the development of an experimental model organism is the possibility to generate different laboratory strains that are homogeneous for a given phenotype or genotype. This can be achieved by artificial selection, where individuals carrying a given extreme trait are selectively bred. Due to its very short life cycle (35–40 days in captivity, i.e. the shortest among all the vertebrates), *Nothobranchius* fish are particularly suited for experimental evolution.

Transgenic *N. furzeri* lines are now available, with fish ubiquitously expressing green fluorescent protein (GFP) under several promoters (Valenzano *et al.*, 2011; Hartmann & Englert, 2012; Allard, Kamei & Duan, 2013). In these lines, the medaka Tol2 transposon system allowed stable integration of the transgene in the host genome, achieving robust expression and transgene persistence over several generations. These proof-of-principle experiments indicate that *Nothobranchius* fish can readily be used to generate transgenic lines that overexpress a construct of interest and therefore as a subject for large-scale genetic screening. Current work is aimed at further developing the transgenesis toolkit in this species and applying the recently developed transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) systems (Zu *et al.*, 2013; Sung *et al.*, 2014) to introduce loss-of function mutations and perform site-specific genome editing.

Following the example provided by the zebrafish community, not only targeted, but also ethyl methanesulfonate (EMS)- and/or N-ethyl-N-nitrosourea (ENU)-induced random mutagenesis could be a powerful future resource to generate a large set of genetic mutant *N. furzeri* strains. Since *Nothobranchius* species are shorter lived than zebrafish and medaka, these mutants could be screened for increased survival or delayed onset of ageing biomarkers and potentially used to identify novel genomic regions that modulate vertebrate survival and ageing.

Overall, the advancements in *Nothobranchius* transgenesis strongly promote the development of these fish as new powerful genetic models for several research fields, including developmental biology and ageing. The possibility of generating transgenic lines rapidly, due to the extremely short life cycle for this vertebrate, makes these fish extremely powerful model systems and we expect growing interest from the scientific community in the future.

(3) Inbred lines

Since its discovery in 1968, *N. furzeri* has been raised in captivity and continuously bred by brother–sister matings, undergoing several bottleneck events due to population shrinkage. Currently, the descendants of the original *N. furzeri* population captured in the Sazale Pan in the Gonarezhou National Park in Zimbabwe constitute the GRZ (Gonarezhou) strain (Table 1). This is the strain that the large majority of laboratories use for experimental work, and currently is the shortest lived. Based on results obtained from genotyping with microsatellite markers, this strain is highly inbred, with females completely inbred (100% homozygosity in 152 tested microsatellite markers) and males heterozygous exclusively at the sex-linked markers (Valenzano *et al.*, 2009).

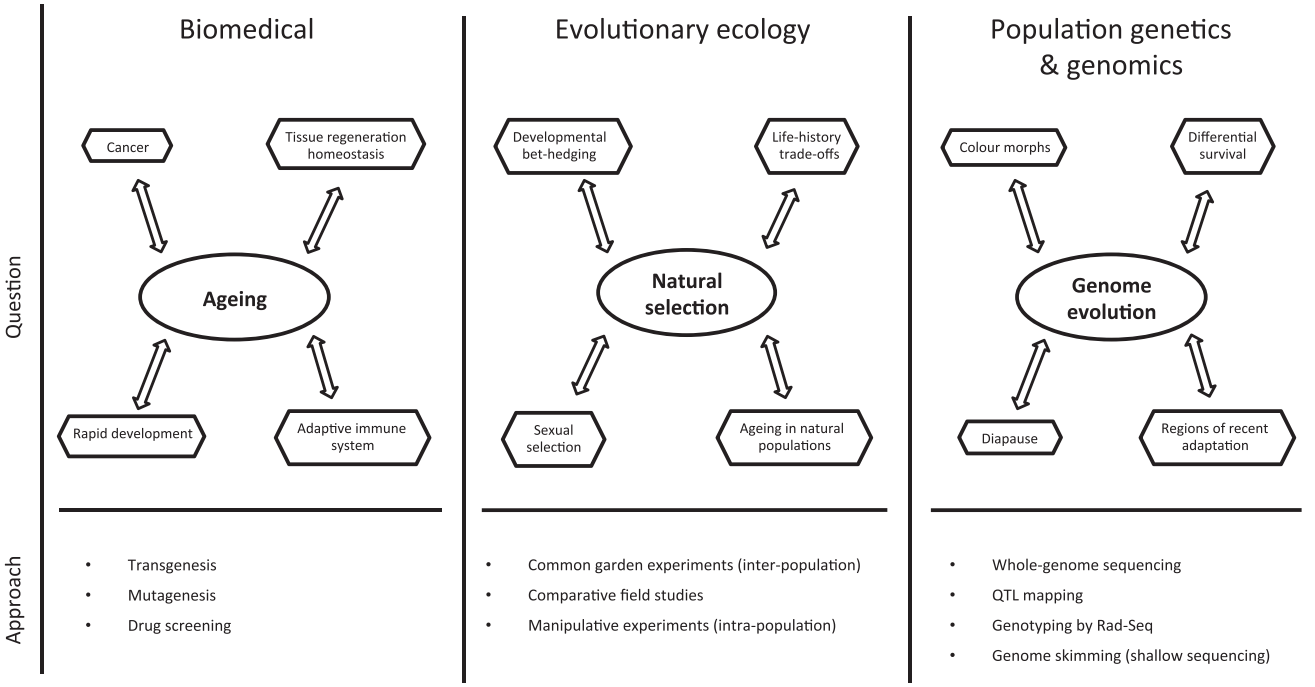

Fig. 6. Schematic overview of research agendas in three branches of biology, the general questions that can be studied using *Nothobranchius* species, and their methodological approaches. QTL, quantitative trait loci; Rad-Seq, restriction site associated DNA markers sequencing.

The genetic homogeneity of the GRZ strain was further confirmed by analysis of single-nucleotide variations (Reichwald *et al.*, 2009; Kirschner *et al.*, 2012). The availability of a highly inbred and viable strain provides a reference homogeneous genetic background for a collection of site-specific genetic mutants, which will help to associate phenotypes to a specific genetic region. Additionally, several other *N. furzeri* strains collected from the wild in 2004 and 2007 are undergoing progressive inbreeding, including the strains MZM0-403, MZM-0410, MZM0-703 (Table 1), which have been bred for more than 25 generations, including specific brother–sister matings. Preliminary observations show that loss of heterozygosity in these strains is not reducing their average survival in captivity as well as their fecundity, indicating an intriguing resilience to the effects of inbreeding in this species as opposed to zebrafish where a lack of genetic homogenous lines represents a hurdle for precise genetic mapping (Guryev *et al.*, 2006; LaFave *et al.*, 2014). The availability of several inbred strains will additionally permit the generation of recombinant inbred lines, which will help in the identification of regions of the genome associated with phenotypes that differ among strains. Finally, inbreeding simplifies genome assembly and efforts in this direction are currently ongoing in several laboratories.

XII. CONCLUSIONS

(1) *N. furzeri* and other *Nothobranchius* species represent a unique model group, both for ecology and evolution and for biomedical research (Fig. 6). The scientific community working on *Nothobranchius* species is expanding and new laboratories are establishing suitable facilities.

(2) *Nothobranchius* spp. can be collected in the wild and kept in the laboratory under standardized conditions, allowing separation of genetic and environmental components of particular life-history traits, including lifespan. There is increasing knowledge regarding genetic differentiation among individual populations of *N. furzeri*, *N. kadleci*, *N. pienaari* and *N. orthonotus* from Southern and Central Mozambique. Several species are distributed throughout a continuous gradient of aridity, forming exceptionally strong tests of the evolution of particular life-history traits.

(3) There are growing data on demography, population genetics, habitat conditions and ecology of wild populations. This is a unique situation compared to other model taxa in biomedical sciences, including *Caenorhabditis elegans* and *Danio rerio*. Such background information should enable us to link particular findings into the natural history of the study species and provide additional, evolutionary insights.

(4) Diapause is the state of developmental arrest where embryos become strongly resistant to external factors and can survive for extended periods of time. This physiological state is rare in vertebrates and provides a unique opportunity to explore the genetic, biochemical and physiological bases associated with developmental arrest in vertebrates. Initial studies demonstrated that diapause can affect posthatching life history and can be manipulated experimentally. Molecular mechanisms

that control diapause are of particular interest as studies in *C. elegans* have shown that some of the genes controlling diapause also control lifespan [e.g. the insulin-like growth factor 1 (IGF-I)/insulin pathway] (Kenyon, 2011). Therefore, characterization of the genetic pathways controlling diapause in *N. furzeri* may reveal novel pathways controlling vertebrate ageing.

(5) Pharmacological, dietary and lifestyle interventions modify lifespan and ageing rate in several species of *Nothobranchius*. Given the shared metabolic and cellular pathways with other vertebrates, including mammals, the short lifespan of *Nothobranchius* makes this taxon exceptionally suited for large-scale testing of non-genetic interventions on ageing and pilot screening of large sets of potentially applicable chemicals. There is growing interest in studying the effects of small molecules on ageing-related dysfunctions, but the increasing costs of mouse husbandry pose a serious limitation for the feasibility of these types of experiments. The lower costs of *Nothobranchius* husbandry, together with its short lifespan, make it a promising system for these types of investigations.

(6) Teleost fish are capable of remarkable regeneration of tissues including the retina, brain, fins, heart and kidney. All these tissues have limited regeneration capability in mammals. The zebrafish is currently favoured in attempts to define the molecular pathways that allow tissue regeneration. Data on effects of ageing on regeneration in zebrafish are controversial: one study reported no effects of ageing on regeneration of fins and heart (Itou *et al.*, 2012), while another reported impaired neuronal regeneration in old fish (Edelmann *et al.*, 2013). *N. furzeri* represents a new model to investigate how ageing impacts on the regenerative properties of multiple tissues.

(7) Evolutionary genomics, including genomic and transcriptomic correlates of differences in lifespan and life-history traits is another field for *N. furzeri* application. The genome of *N. furzeri* has been sequenced and assembled and will likely be released in the near future. *Nothobranchius* fish therefore offer a unique possibility to study how the genome evolves under differential extrinsic mortality patterns and parallel evolution in sympatric clades (Terzibasi Tozzini *et al.*, 2013). To date, only initial mapping of lifespan and two ageing-related traits has been performed. These traits will be mapped at a higher resolution. However, there are several other traits that differ among *N. furzeri* populations and there is an opportunity to analyse different *N. furzeri* clades. Finally, studies of gene expression offer large numbers of traits that can be used in expressed QTL (eQTL) approaches to investigate the genetic architecture of age-dependent gene regulation.

(8) The evolution of *Nothobranchius* colouration is an active field of study and has the potential not only to provide insights into the molecular and evolutionary mechanisms responsible for morphological differentiation within a species, but can also reveal fundamental molecular clues about the mechanisms involved in the onset of pigment aberrations, including melanoma.

(9) The very short generation time of *N. furzeri* compared to zebrafish makes this species attractive for transgenic models of adult phenotypes. The possibility of generating transgenic lines rapidly may attract a large community of scientists not exclusively focused on ageing research. In particular, *N. furzeri* may become a useful organism to model progressive diseases with late onset that are expensive to study in standard models such as the mouse or zebrafish.

(10) The development of general guidelines for husbandry of *Nothobranchius* spp. in a laboratory setting will help an increasingly larger community to adopt this fish as a novel model system and will help the establishment of basic reference protocols for this promising new group of experimental organisms.

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XIV. REFERENCES

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